

EXPERIMENTAL CELL RESEARCH



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SUBMICROSCOPIC STRUCTURE OF MEIOTIC CHROMOSOMES DURING PROPHASE

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IN surveying the published reports on chromosome submicroscopic organization it becomes apparent that there is still a wide field to investigate on this subject. As noted before [6, 4] the electron microscope has failed to corroborate some facts of the chromosome structure which, after being the subject of extensive controversies, were admitted and became fundamental for modern cytogenetists.

The chromosomes have been currently described by electron microscopists as being integrated by submicroscopic filamentous units [9, 10, 5], but no reference has been made in the early reports on the existence of differentiated structures within the chromosomes recalling the chromonema of optical researchers.

The first notice of the existence of a heterogeneous structure in the chromosomes is due to Moses [6] who reported the existence of a central core showing an organized fine structure in the prophasic chromosomes of the primary spermatocytes of *Cambarus clarkii*. As described by Moses the chromosome central core would be a dense rod embedded in thin concentric layers.

Pigeon, cat and man spermatocytes were studied by Fawcett [4]. He found a similarly organized core in the prophasic chromosomes, but instead of a single rod he described it as a ribbon-like structure formed by three parallel fibrils, the two external fibrils being denser and thicker than the inner one.

The present paper reports on similar structures found in the early prophase of the primary spermatocyte of the rat (*Mus norvegicus albinus*), fish (*Cnesterodon decemmaculatus*) and opilionoidea (*Heteropachylloidelus robustus*).

MATERIAL AND METHODS

Testicular tubes were removed from the living animal (white rats) and fixed in 1 per cent osmium tetroxide solution buffered at pH 7.6 with veronal acetate as

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recommended by Palade [7]. After the usual embedding in *n*-butylmethacrylate, sections were cut with the Porter-Blum microtome for thin sectioning [8]. Examination was carried out with an R.C.A. EMU electron microscope. Pictures were taken at original magnifications of 10,000 to 13,000 diameters and enlarged as convenient.

Eventually, thick sections ($0.5\ \mu$) of the same material were stained according to the Feulgen method after solving the methacrylate embedding with acetone or chloroform (this step does not take more than $\frac{1}{2}$ minute).

OBSERVATIONS

Electron micrographs of prophasic nuclei show a highly organized structure resembling the ones found by Moses and by Fawcett. These structures were found existing in nuclei in which the optical examination of Feulgen-stained sections did not yet show the presence of chromosome bodies. They are ribbon-like elements constituted by a thin medial filament ($120\ \text{\AA}$ thick) accompanied by two dense, thick threads of about $500\ \text{\AA}$. Each thread is separated from the medial one by a clear space of about $200\ \text{\AA}$.

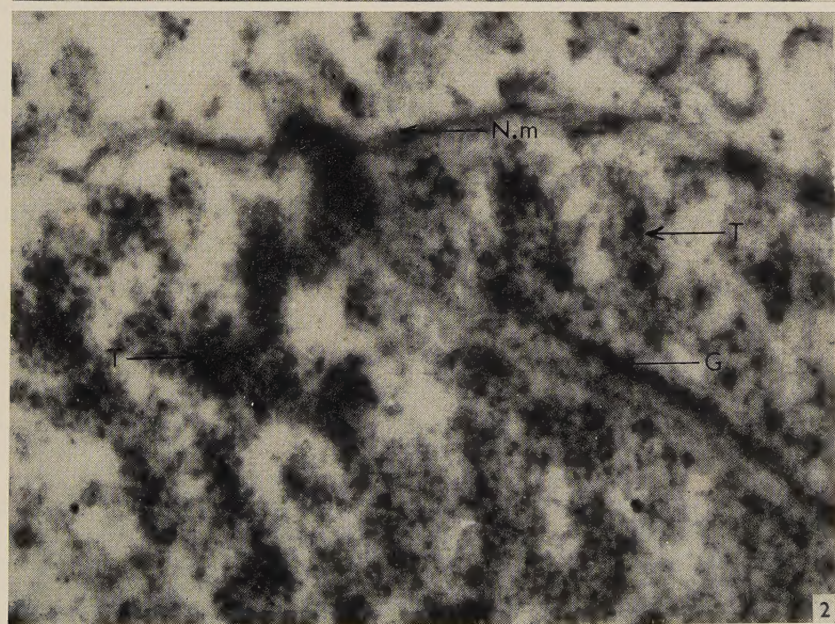
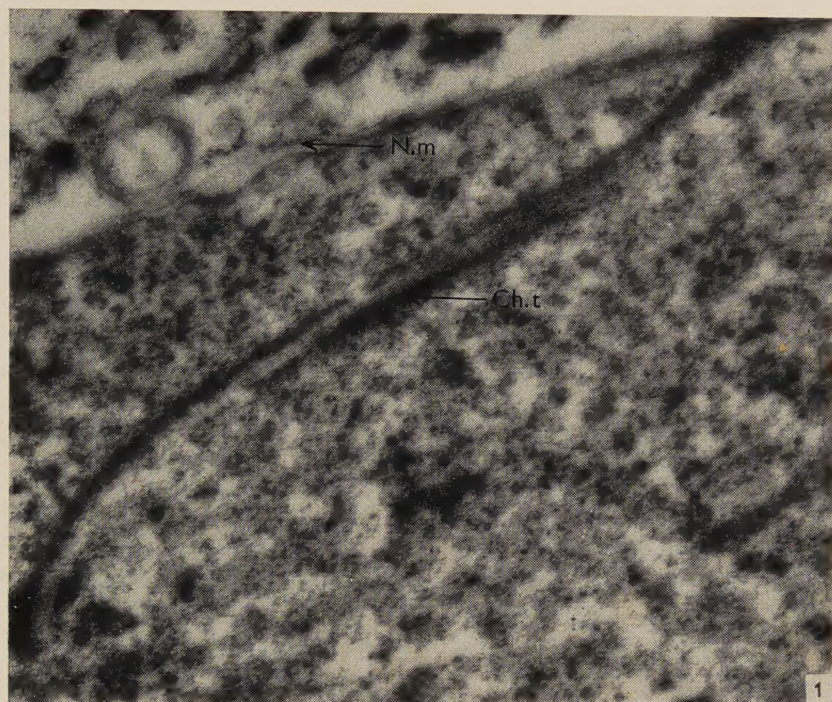
The medial filament seems to possess a fine structure but the individual elements composing it match the resolution power of the electron microscope. In some micrographs it has the appearance of a double-lined structure. The elemental components of the outer threads are filaments of 80 to $100\ \text{\AA}$ wide and indefinite length. The cross sections of the group show that each component is a single independent unit.

A close relationship of these organized groups to the nuclear envelope has been found. In most of the observed nuclei, at least one or two of these linearly arranged structures were found attached to the nuclear membrane. At the contact zone the density of the two membrane sheets appears greater than usual.

In some cases a parallel array of filaments bounding each side of the thread group has been found. This was particularly observed at the membrane attachment point. This type of element coexists in the same nucleus with still uncoated groups. A number of observations proved that at a later stage of prophase each group of threads becomes completely surrounded by chromosomic material. In such cases no particular order of the material was

Fig. 1.—Electron micrograph of a group of filaments found still uncoated by chromatic material. The small condensations of material observable on several places of the nucleus seems to be unrelated to the group. *Nm*, nuclear membrane; *Cht*, chromosomic thread. $\times 52,012$.

Fig. 2.—Group of filaments *G* surrounded by a thread-like mass *T* having the appearance of nucleolus. It cannot be stated if the latter corresponds to nucleolar or to chromosome material. *Nm*, nuclear membrane. $\times 44,580$.



observed. The Feulgen method applied to thick sections detected thin filaments about $0.2\ \mu$ wide having a disposition similar to the one observed in the electron micrographs of the same nuclei. It should be stressed that many of the parallel groups of filaments found in the micrographs are about the same width ($0.18\ \mu$). Of course it cannot be stated whether the filaments are wholly composed of Feulgen positive substances or not, because a light external coating of Feulgen positive material could be responsible for the optical images. In many cases, as in Fig. 2, the group of threads was found embedded in a mass of granular material arranged in thick filaments similar to the ones described by optical examination [3] or by electron microscopy [1, 2] as forming part of the nucleolus. A careful examination was made in order to clear out the cases in which the surrounding material is of chromosome or nucleolar nature. Sometimes at this stage the elemental components of both structures are quite similar in morphological appearance. In such cases Feulgen positive masses about the threads were not detected, but it is not always possible to examine with the electron microscope the same nuclei observed in the stained thick sections because many of them become masked by the grid wires. In such cases the compactness of the material or its thread-like tangled arrangement are the only useful differential character available in the micrographs.

It can be seen in the pictures that the above-mentioned structures do not follow a straight but a rather wavy course. Besides the undulating course followed by the whole group, it was observed that a frequent change of plane of each filament takes place within the group. This can be easily seen in micrographs showing a long segment of them; furthermore, in most parts of the micrographs the external threads of each group appear of unequal length. (Fig. 4 shows one of the external threads contacting the nuclear

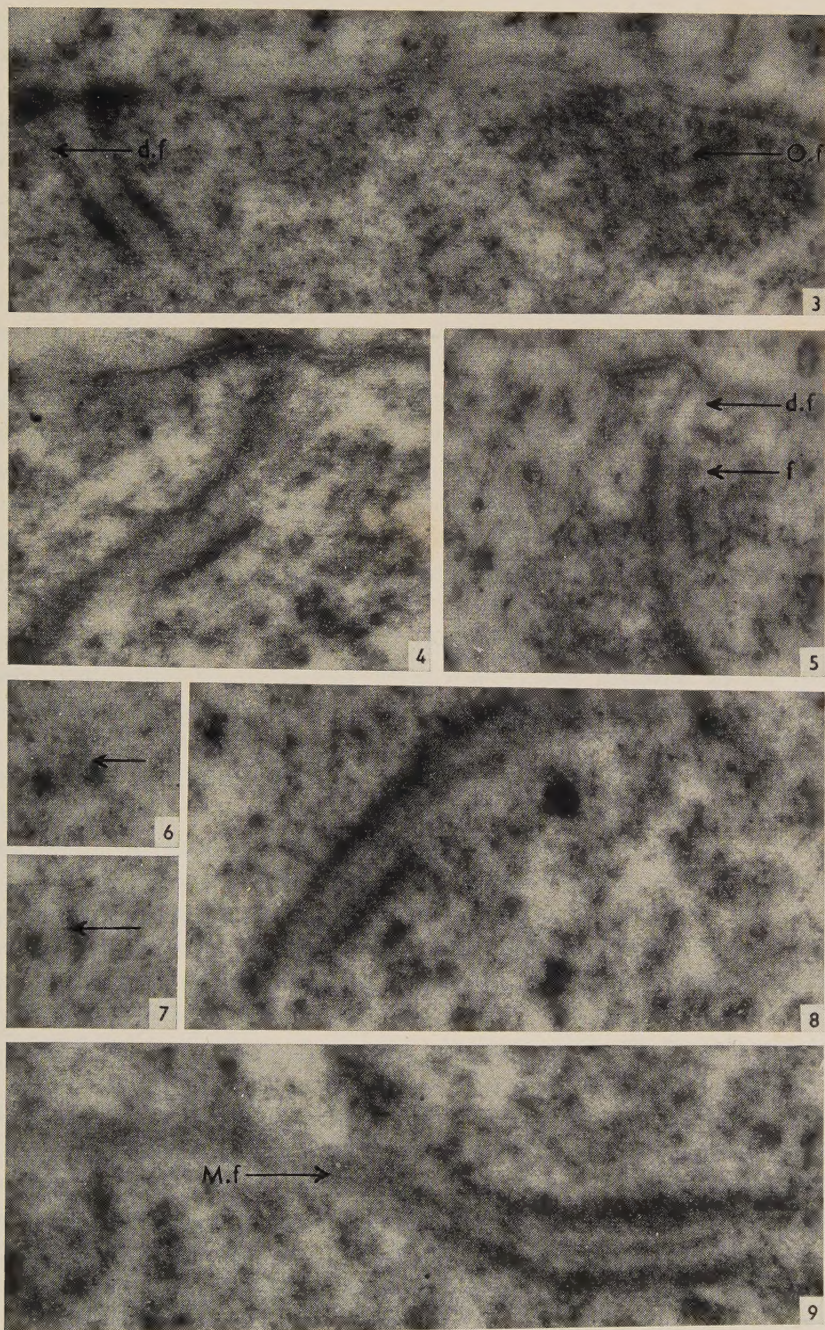
Fig. 3.—Picture of two groups of threads, one of them (*of*) already embedded in chromosome material. The one seen at the left of the picture, *df*, shows a double-lined appearance. $\times 44,580$.

Fig. 4.—It is shown that one of the external threads of the group is shorter than the other, which demonstrates the change of plane mentioned in the text. $\times 44,580$.

Fig. 5.—The group of threads seen in this micrograph shows condensations of material at both sides of the group. Just beneath the nuclear membrane it is seen that one of the threads (*df*) has a double-lined structure whose components are similar to the ones seen arranged parallel outside the threads. $\times 44,580$.

Figs. 6 and 7.—Cross sections (marked by arrows) of the thread-like structures showing their components as independent units. $\times 44,580$.

Figs. 8 and 9.—High magnification pictures of two groups of filaments found in a same nucleus. The one seen in Fig. 8 shows the existence of an apparent continuity between the material forming the external threads and the slightly condensed material lying beside them. The long band seen in Fig. 9, *Mf*, seems to be continued by the medial filament of the group. $\times 78,018$.



membrane, while the other one appears interrupted by the section.) Thus it may be supposed that the plane variations of the threads are considerably frequent along their course.

In the pictures taken for this report it could not be determined if this spatial relationship between the components of the groups is related to the known structural pattern described for the chromonemata. Nevertheless, the images obtained (Fig. 1) suggest the probable existence of wide helicoidal turns which would support this concept.

It must be stressed again that these senuclear components have been encountered in different evolutive stages. Ordering these stages in accordance to the morphological appearance of the grouped threads it can be said: (a) that the beginning of prophase is likely to be characterized by the presence of units of this class which are constituted only by thin elemental filaments; (b) in a following phase the elemental filaments increase in number and granules become attached or intermixed with them; these two stages are difficult to detect by optical means; (c) finally, the ribbon-like groups become embedded in Feulgen positive material which is forming the bulk of prophasic chromosomes.

No structures of such a class could be found in gonial nuclei of testicular tubes of young animals (rat). They were found appearing only during the preparation of meiosis, but not in later stages (metaphase, etc.) or in somatic mitosis of other cells.

The spermatocytes nuclei of *Cnesterodon decemmaculatus* and of *Heteropachylloidelus robustus* showed structures similar to the one described above. However, some differences must be noticed. *Cnesterodon* spermatocytes do not show in all prophasic stages the central filament described in rat spermatocytes. It seems that this filament disappears before chromatin material begins to assemble around the group of filaments.

Heteropachylloidelus spermatocytes show that the filaments of the group fuse during earliest prophase, in such a way that the group is transformed in a single thread. The process of chromosome formation for both species is in other respects similar to the one described for rat spermatocytes.

DISCUSSION

The organized structures reported here are of the type described by Moses or by Fawcett [6, 4]. Nevertheless, it should be pointed out that the observations made for the present paper differ in some points from Moses' findings, being instead in full accordance with the facts described by Fawcett.

Leaving aside some small structural differences, one can conclude that this type of structure must be of frequent occurrence in meiotic organization of animal species. Recent observations made in our laboratory add two new species (*Heteropachylloidelus robustus*, spider, and *Cnesterodon decemmaculatus*, fish) to the ones in which such structures were seen; the structural differences found in these species are not considered relevant. It is not easily understood why similar structures cannot be found in somatic mitosis, or why they do not exist or remain undetected during the following stages of the meiotic cycle.

It is obvious that a full study must be carried out on the prophasic stages of meiotic chromosomes, chiefly trying to establish a better correspondence between the optical knowledge of the chromosome organization and electron microscope findings specially in regard to the behaviour of this structure during the synaptic stage.

The admission that this structure may correspond to the chromonema is perhaps not yet well founded, because it has not been demonstrated in mitotic chromosomes in which the chromonema can be clearly seen by optical procedures. As sufficient information about these problems has so far not been gained, they remain still unsolved.

In the case of meiotic chromosomes it is probable that the whole structure is being organized around thin filaments of the same order as the medial one. Chromatic material (DNA) may coat the external threads or may mix among the elemental units composing them while the medial filament apparently remains uncoated.

Some of the pictures shown in this paper support this view (cases in which the external threads show in part a double-lined structure). Also the medial filament may not be constant. The recent observations made on *Cnesterodon* spermatocytes only show the external filaments, but as only few specimens of these genera have been examined, it would be premature to draw any conclusion.

SUMMARY

The existence of well differentiated structures in prophasic nuclei of rat primary spermatocytes has been demonstrated. These structures consist of ribbon-like groups of three threads; two external thick and dense (500 Å wide); one medial thin and light (120 Å wide). The external ones run equidistant to the medial filament (with a distance of 200 Å between them). The cross sections of the groups show that each filament is an independent unit.

As the position of the external threads was seen changing from side to side of the medial one, it is suggested that they may have a wide helicoidal course.

A number of them were seen contacting or attached to the inner sheet of the nuclear membrane. They were found existing only in the preparatory stages of meiosis.

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HEMOLYSIS OF INDIVIDUAL RED BLOOD CELLS

AN INTERFEROMETER MICROSCOPIC INVESTIGATION

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HEMOLYSIS, its cause and the mechanism by which hemoglobin leaves the red blood cell has now been under investigation for about a hundred years. During this time vast knowledge has accumulated concerning the properties of the erythrocyte and the events prior to, accompanying and following hemolysis. However, there is at present no unified picture which can explain the mechanism by which the hemoglobin is liberated.

The almost regular prolytic increase in cell volume [21], the fact that erythrocytes behave as almost perfect osmometers [13] and the impression obtained when erythrocytes are seen to hemolyse under the microscope [3] are some of the observations which have led to the so-called balloon theory [22]. According to this theory the red blood cell is made up of a cell membrane containing a solution of hemoglobin, and hemolysis is primarily caused by an increase in the intracellular osmotic pressure, leading to a rupture of the membrane and ejaculation of the cell content.

There are, however, several observations that argue against such a simple explanation. If hemolysis is supposed to be caused by a rupture of the cell membrane, it should occur when the membrane is stretched beyond a certain critical limit. Since it has been found that the critical surface area at which hemolysis starts differs when different lytic methods are used [22], it seems evident that at least sometimes the elastic properties of the membrane change during the prolytic phase. At the same time the membrane "permeability" to hemoglobin might change as well, and we are no longer convinced that hemolysis is caused by a rupture of the cell membrane. Investigations on the properties of the membrane after hemolysis have made the occurrence of a rupture even more improbable. In 1935 Fricke and Curtis [11] measured the electric impedance of red blood cells hemolysed with water. They concluded that "the membrane did persist, although showing evidence of injury". Thus, their results argue against the occurrence of a large rupture of the membrane, but if hemoglobin is assumed to escape through small holes, it is not surprising that the capacity of the ghost is substantially the same as that of the unhemolysed cell [22]. In 1952, Teorell showed that the semipermeable properties of the membrane persist after hemolysis [26]. This makes it, of course, improbable that the cell content escapes through a rupture of the membrane. Electron microscopic pictures [17] of erythrocyte ghosts show that the membrane remains intact, or that "the membrane has a homogeneous coat which appears to be porous after it is washed with distilled water" [16]. These facts militate against the opinion that hemolysis is caused by a rupture of the membrane. This is also stated by Parpart and Ballentine [20] in their

discussion of the molecular anatomy of the red cell plasma membrane. They express the opinion that the hemoglobin escapes through channels uniformly spread over the surface of the cell, and that the diameter of these channels can be greatly and reversibly altered.

In what has been said above, the possible influence of the state of the intracellular hemoglobin has been disregarded. The high concentration of hemoglobin in the red cell interior and the occurrence of stromatin [15], which can form gels at very low concentrations [2], indicate that the liberation of hemoglobin may be greatly influenced by strong intermolecular forces. This is most clearly expressed in what may be called the dissociation theory [17], according to which hemolysis is primarily caused by a loosening up of intermolecular bonds. From investigations on the hydrated red cell membrane, conclusions have been drawn that the membrane is $0.5\ \mu$ thick [23] and made up of a 2 per cent non-hemoglobin protein gel containing hemoglobin and surrounded by a bimolecular layer of lipids [19]. This can be interpreted in the same way as predicted by the dissociation theory, namely that hemolysis is caused by the liberation of hemoglobin molecules from a highly viscous body and that a thin, definite and mechanically resistant membrane, if it does exist, is of secondary importance.

This survey should show that there are different opinions about the ultimate mechanism of hemolysis and that it does not at present seem possible to make a satisfactory synthesis of the theories proposed.

It is believed that if we want more exact knowledge of the mechanism of hemolysis, this can best be obtained by investigating the lysis of individual cells. Such investigations have been done previously [14], although it has not been possible to follow quantitatively the loss of hemoglobin. There are now, however, optical methods available by which the amount of substance in a single cell can be determined, and it has been shown that such a method can be applied to studies on hemolysis [18].

The present paper deals with the technique of recording quantitatively, by means of interferometer microscopy, the loss of hemoglobin from individual red blood cells, and with the general results obtained when hemolysis is caused by different lytic agents.

METHOD

Cinematographic pictures of hemolysing red blood cells were taken through a Cooke-Dyson interferometer microscope [7, 8, 9]. On each picture the optical retardation caused by a cell was determined. From the optical retardation the dry weight of a cell, that is the amount of substance other than water can be calculated. By measuring the optical retardation of a fading cell on successive pictures the velocity at which hemoglobin left a cell was followed quantitatively.

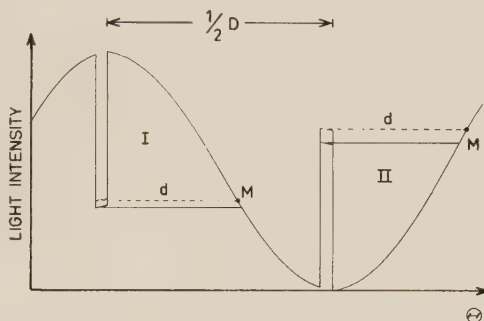
The recording system

The smaller objective of the interferometer microscope, numerical aperture 0.85, was used. The condensor n.a. was 1.3 and the overall magnification was $\text{ca} \times 100$. A high pressure mercury arc was used as a light source, from which the $546\ \text{m}\mu$

band was isolated. Hemolysing cells were recorded on Kodak Tri-X, 16 mm negative film and the film velocity was 8 pictures per second.

The discussion of the Cooke-Dyson interferometer microscope given by Davies *et al.* [5] shows that the microscope can be adjusted so that the phase difference between the two interfering wave fronts varies continuously across the field in view. When monochromatic light is used, the straight interference fringes appear as black and white bands and the optical retardation caused by an object can be determined

Fig. 1.—Because of the continuous variation in phase difference between the two interfering wave fronts, the light intensity varies systematically across the field. The optical retardation caused by an object is proportional to the distance d , and the distance D corresponds to one wavelength optical retardation. Owing to the error of the method, the recorded distance d (continuous line) is too long when an object is situated as at position I (the dotted line indicates the correct distance) and too short when situated as at position II.



by measuring the distance, d , from the object to the corresponding point in the interference bands which has the same light intensity (Fig. 1). In this investigation, the distance, d , was determined with a Zeiss microphotometer in the following way: The film density of the central part of a red blood cell was determined and the slide holding the film was then moved at right angles to the interference fringes with the aid of a micrometerscrew until the corresponding point which had the same film density was found. The photometer slit had the form of a square, the side of which corresponded to ca. $\frac{1}{4}$ of a cell diameter, and was kept constant during the measurements on successive pictures on each cell. The scale of the micrometer screw was in 0.01 mm and the distance, d , for a prolytic cell was of the magnitude 1.6 mm.

The hemolysing system

Since the optical properties of the Cooke-Dyson interferometer microscope requires the use of an immersion fluid, hemolysis always took place between slide and coverslip. In order to avoid "spontaneous" sphering and hemolysis [12], siliconated slides and coverslips were used, when possible. In such cases the erythrocytes do not stick to the glass but are free to move, which must be avoided. It was found that by applying a small amount of silicone adhesive to the siliconated slide, leaving the central part free, and then pressing a siliconated coverslip firmly down before baking, a thin chamber (10–20 μ) could be obtained in which the cells did not move. The blood cell suspension was injected under the coverslip through a groove in the slide and throughout a 5 per cent cell suspension was used. Hemolysis was initiated after the object was focused in the microscope and an image of a number of cells was obtained in the plane of the film.

Osmotic hemolysis.—In order to record osmotic hemolysis a suspension of human erythrocytes in isotonic saline was placed between ordinary slide and coverslip and

focused in the microscope. When optimal light conditions were obtained, hemolysis was started by allowing a small amount of water to run in under the coverslip. When the right amount of water was used, erythrocytes adsorbed on the glass did not move, and hemolysis could be recorded.

Delayed hemolysis in hypotonic glucose solutions.—It has been shown [14] that the length of the prolytic phase of delayed hemolysis in a hypotonic glucose solution is considerably reduced when the temperature is increased. Taking advantage of this, it was possible to adjust the microscope at room temperature and to initiate hemolysis by raising the temperature. Experiments were performed in the following way: A suspension of oxygenated human red blood cells in a 2.2 per cent glucose solution was injected into a thin chamber made up of siliconated glasses as described above. Through the groove in the slide a specially constructed thin thermoelement was placed under the coverglass, just outside the field in view. The temperature was raised to $38 \pm 2^\circ\text{C}$ by means of a piece of metal in the form of a horseshoe and through which thermostatically regulated water was run. The "horseshoe" was placed around the objective in contact with the coverglass and in such a way that contact with the thermoelement was avoided.

Immune body hemolysis.—Immune body hemolysis of sensitized sheep erythrocytes was recorded between ordinary glasses, using four times as much complement as the amount required to give 100 per cent hemolysis after 30 minutes at 37°C . After that the sensitized erythrocytes were focused, the suspension of complement was allowed to run in, and hemolysis was recorded after 3–5 minutes.

Hemolysis by a photodynamic active substance.—The most convenient hemolysing system for this investigation was a system where a photodynamic active substance, rose bengal [1], was used. Such a system makes it possible to initiate the hemolysis in a medium of desired composition simply by illuminating it with ultraviolet light. The results presented here were obtained with 1 mg per cent and $\frac{2}{3}$ mg per cent solutions of rose bengal and oxygenated human red blood cells.

Calculation of the intracellular concentration of hemoglobin

The recorded optical retardation (ϕ_{exp}) is the difference between the optical retardation caused by the object and that caused by an equal thickness of the medium. In order to simplify the following discussion, the expression for the optical retardation caused by a red blood cell is divided into two components. One of these is the optical retardation caused by the intracellular hemoglobin and the other one is the optical retardation caused by non-hemoglobin cell material. The following expression is then obtained:

$$\phi_{\text{exp}} = \phi_{\text{Hb}} + \phi_{\text{non-Hb}} - \phi_{\text{med}} \quad (1)$$

Table I shows the relative magnitudes of the optical retardations caused by hemoglobin, non-hemoglobin cell components and the surrounding medium. These calculations were done from figures given by Ponder for the red cell composition [22] and from values of specific refractive increments

TABLE I

	$g \times 10^{-12}$ per cell	Specific refractive increment	Per cent of total cell optical retardation
<i>Erythrocyte</i>			
Hemoglobin	29	0.0019	94.3
Mineral	0.6	0.0014	1.4
Protein	0.8	0.0019	2.6
Lipid	0.7	0.0014	1.7
Total			100.0
<i>Medium</i>			
Isotonic saline	0.78	0.0017	2.2

[5, 25]. It is seen that ϕ_{Hb} is equal to ϕ_{exp} within 5 per cent if the surrounding medium consists of isotonic saline. If the medium is made up of a hypotonic glucose solution, contains a certain amount of liberated hemoglobin or if the temperature is altered, this figure will change slightly, but can always be regarded as constant during the fading of a single cell. As a first approximation we can write:

$$\phi_{\text{exp}} = \phi_{\text{Hb}}. \quad (2)$$

ϕ_{exp} is obtained from measurements on the photographs (see Fig. 1) according to equation

$$\phi_{\text{exp}} = \frac{d}{D} \lambda, \quad (3)$$

where d = the distance on the film from the object to the corresponding line in the interference fringe which has the same film density; D = the distance corresponding to an optical retardation of one wavelength and λ = wavelength of the light used.

The concentration of hemoglobin can be calculated from equation

$$\phi_{\text{Hb}} = a \cdot l \cdot c, \quad (4)$$

where a = specific refractive increment of hemoglobin; l = thickness of the cell; and c = concentration of hemoglobin.

Combining (2), (3) and (4) we get

$$d = \frac{D a l}{\lambda} c. \quad (5)$$

Owing to the experimental conditions D and λ are constant during hemolysis. Changes in specific refractive increment with variations in the concentration of hemoglobin are too small to be of importance [4]. Hence α can be regarded as a constant. According to studies of the hemolysis of individual erythrocytes [22], no changes can be observed in form or cell volume after the initial phenomenon of spherocytosis. Nor have any such changes been observed in this investigation. It can therefore be assumed that l is also constant during the fading of a red blood cell. Equation (5) can then be rewritten as

$$d/d_0 = c/c_0, \quad (6)$$

where d_0 and c_0 refer to the polytropic values.

RESULTS

The hemolysis of 27 red blood cells was followed from the onset of hemolysis until 50 to 90 per cent of the hemoglobin was lost. In Fig. 2 examples are given, showing the hemolysis of individual cells and the variation of the experimental values. The curves were drawn with the aid of the method of moving averages, each step embracing 3 values. Hemolysis was caused by different methods and by each method the following number of cells were investigated: Photodynamic hemolysis I, $\frac{2}{3}$ mg per cent rose bengal (4 cells); II, 1 mg per cent rose bengal (9 cells); III, immune body hemolysis (8 cells); IV, delayed hemolysis in a 2.2 per cent glucose solution (3 cells); V, osmotic hemolysis (3 cells). The mean curves (Fig. 3), describing the fading of erythrocytes hemolysed by each method, were calculated from the

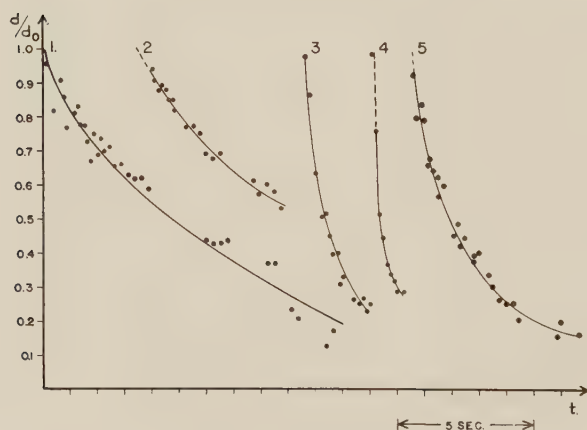


Fig. 2.—Examples of the recorded hemolysis of individual cells, showing d/d_0 [(optical retardation at time t)/(optical retardation at the onset of hemolysis, $t = 0$)] as a function of time.

individual curves by determining the mean time used for a decrease of the d/d_0 values from 0.9 to 0.8, 0.7, 0.6 and so on.

It was found, for all erythrocytes investigated, that the onset of hemolysis was abrupt and that there was a continuous decrease of the velocity at which the hemoglobin left a cell. When the fading time was short the impression was obtained that the cell content was ejaculated.

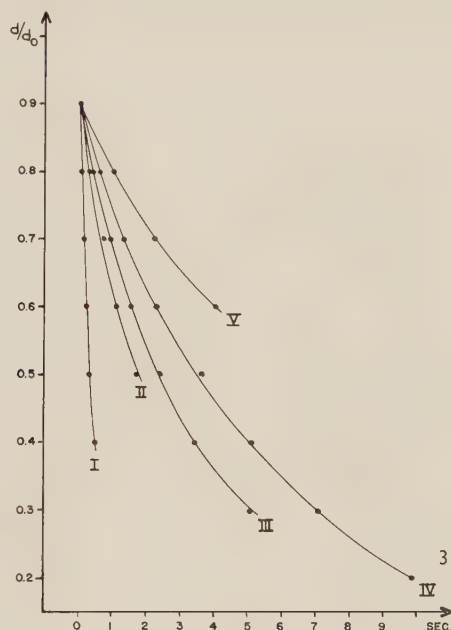


Fig. 3.—Mean curves showing the hemolysis caused by different methods. Since the actual zero time was obtained by extrapolating the individual curves to the onset of hemolysis (see Fig. 2, examples 2, 4 and 5), the time scale was in this figure chosen to run from $d/d_0 = 0.9$. The curves show the hemolysis caused by I, $\frac{2}{3}$ mg per cent rose bengal; II, 1 mg per cent rose bengal; III, immune body hemolysis; IV, delayed hemolysis; V, osmotic hemolysis.

DISCUSSION

As a basis for the following discussion of the experimental results, the assumptions made by Fricke in his paper "The rate of escape of hemoglobin from the hemolysed red corpuscle" [10] were chosen. This was done since Fricke's assumptions are essential and can be expressed in a convenient mathematical form, which makes it possible to calculate the correlation between the theory and the results obtained here. If the results do not fit the theory, the deviations can be analysed and interpreted. The assumptions are:

- (1) Hemoglobin is contained in solution within a surface membrane (and the resistance to liberation of hemoglobin is at the surface of the cell).

- (2) The permeability of the membrane to hemoglobin is constant during hemolysis.
- (3) Hemoglobin is transferred across the cell membrane through free diffusion.
- (4) Fick's law applies with a constant value of the coefficient of diffusion.

If these assumptions are valid, the formula given by Fricke should hold:

$$c = c_0 e^{-3\mu t/r}, \quad (7)$$

where c = concentration of hemoglobin in the cell at time t ; c_0 = concentration of hemoglobin at the onset of hemolysis ($t = 0$); r = radius of the cell, and μ = permeability of the membrane to hemoglobin defined as the amount of hemoglobin passing through unit area in unit time per unit difference of concentration between inside and outside solutions.

However, it was found that $\log d/d_0$ was not a linear function of time, and that the deviation from the linear decrease of $\log d/d_0$ was larger than could be explained by the 5 per cent error introduced by eq. (2). Since it is generally known that a red blood cell does not lose all its hemoglobin, the first of the assumptions made above was modified.

A new attempt to correlate Fricke's equation to the experimental results was made by stating that only part of the intracellular hemoglobin is free to diffuse, whereas the residual hemoglobin is fixed to the red cell stroma. Using this new assumption, the recorded distance, d , was related to the intracellular concentration, c , of free diffusible hemoglobin by the formula:

$$d - \alpha d_0 = A c, \quad (8)$$

where d is proportional to the total concentration of cell material and αd_0 represents the concentration of nondiffusible hemoglobin and in addition contains the error introduced by eq. (2). A is a constant (see eq. (5)).

By combining (8) with the formula given by Fricke we get

$$d/d_0 - \alpha = (1 - \alpha) e^{-kt}. \quad (9)$$

This equation may be used in order to find values of α and k , giving the best possible correlation between theory and experiment. If connected values of d/d_0 and t , one pair of values found at time t_1 and the other pair found at time t_2 , are inserted in (9), the two equations obtained can be combined to give

$$\frac{d_1/d_0 - \alpha}{d_2/d_0 - \alpha} = e^{-k(t_1 - t_2)}. \quad (10)$$

Keeping the time interval $(t_1 - t_2)$ constant we can write

$$d_1/d_0 = K d_2/d_0 + \alpha(1 - K), \quad (11)$$

where $K = e^{-k(t_1 - t_2)}$ is a constant.

From the plot of experimentally obtained values of d_1/d_0 against d_2/d_0 , regression lines were calculated by ordinary statistical methods. Since both d_1/d_0 and d_2/d_0 can be regarded as the independent variable, two

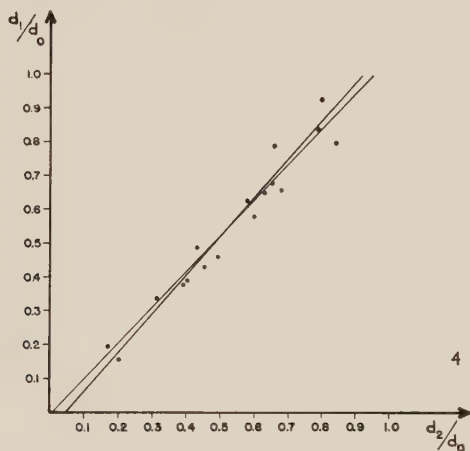


Fig. 4.—Plot of successive values of d/d_0 against each other for one red blood cell. Two regression lines were calculated from each plot.

regression lines were calculated from each plot (Fig. 4). In most cases the two lines coincided well, which indicates a high coefficient of correlation. It seems reasonable to assume that the bisectrix to the two lines represents the plotted values better than any of the regression lines. If the hemolysis of a red blood cell can be described by eq. (9), the equation of the bisectrix should be identical with eq. (11). However, the mean value of α , calculated from the equations of the bisectrices, was 0.41. If such a value is correct it means that erythrocytes lose less than 60 per cent of their dry weight during hemolysis. This is rather improbable since 90 per cent is a generally accepted figure. In addition it was found that when the hemolysis of a red blood cell was followed long enough, the d/d_0 value recorded last was generally lower than the calculated value of α (Fig. 5). Because of these facts, the conclusion was drawn that the hemolysis of an erythrocyte cannot be described by eq. (9). Consequently, hemolysis cannot be explained by a free diffusion of hemoglobin through a cell membrane having a constant permeability to hemoglobin.

In order to find an alternative explanation to the mechanism of hemolysis,

the experimental results were treated in another and more simple way. Since d/d_0 represents the relative dry weight of a cell (dry weight at time t /dry weight at the onset of hemolysis, ($t = 0$)), the velocity at which the d/d_0 value decreases also represents the velocity at which hemoglobin leaves a cell. The velocity of hemolysis was therefore calculated by determining the time used for a decrease of the d/d_0 values from 0.9 to 0.8, from 0.8 to 0.7, and so on. These determinations were done from experimental curves drawn with the aid of the method of moving averages (see p. 14). By

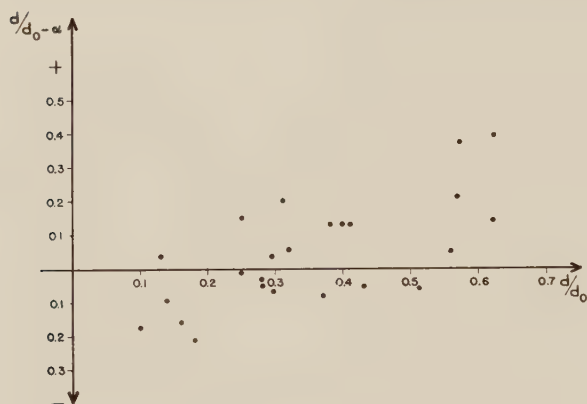


Fig. 5.— d/d_0 here refers to the last recorded d/d_0 value of each cell and α was obtained as described on p. 17. It is seen that the last recorded values of d/d_0 are lower than the calculated values of α , ($d/d_0 - \alpha < 0$) for 73 per cent of the cells, when the hemolysis was followed to d/d_0 values lower than 0.30.

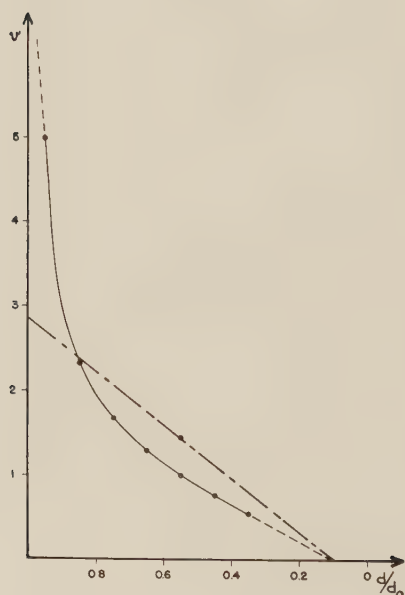


Fig. 6.—The mean velocity, v , of the hemolysis of 21 cells, expressed relative to the velocity at $d/d_0 = 0.55$, as a function of the relative optical retardation d/d_0 of the cells (continuous curve). The broken line shows the velocity of hemolysis, if the hemoglobin leaves a cell by free diffusion and hemolysis takes place within the same time in both cases. The slope of the curves is proportional to the membrane permeability to hemoglobin and to the surface area through which the hemoglobin leaves the cell.

TABLE II

Mean difference between velocities	$\bar{x} \pm \epsilon_{\bar{x}}$	σ	(n)
$v_{0.85} - v_{0.75}$	0.65 ± 0.11	0.48	(21)
$v_{0.75} - v_{0.65}$	0.41 ± 0.05	0.23	(21)
$v_{0.65} - v_{0.55}$	0.27 ± 0.04	0.19	(21)
$v_{0.55} - v_{0.45}$	0.24 ± 0.04	0.15	(16)
$v_{0.45} - v_{0.35}$	0.22 ± 0.04	0.14	(11)

$\bar{x} \pm \epsilon_{\bar{x}}$ = Mean $\pm$ error; σ = stand. dev.; (n) = number of cases.

putting the time required for a decrease of d/d_0 from 0.6 to 0.5 equal to an arbitrary unit, 0.1, for all cells investigated, the mean relative velocity, v , was calculated at d/d_0 values of 0.85, 0.75, 0.65, and so on. The resulting curve is shown by Fig. 6. The velocity of hemolysis at $d/d_0 = 0.95$ was calculated when the individual curves were extrapolated to the onset of hemolysis. Six cells were not followed as long as to $d/d_0 = 0.5$ and were therefore excluded.

It is seen from Fig. 6 that the velocity at which hemoglobin leaves a cell is relatively high at the beginning of hemolysis, and that at the end of hemolysis the velocity decreases in an approximately linear manner. No exception from this rule was observed. The deviation from the linear decrease at the beginning of hemolysis is also obvious from Table II. Extrapolation of the curve shown in Fig. 6 to the end point of hemolysis ($v = 0$) shows that the dry weight of the ghost cells was found to be 10 per cent of the dry weight of the intact cells, which is the figure generally accepted.

Error

At an early stage of this investigation it was observed that the light intensity of a red blood cell was never as high as the light intensity at maxima of the field. There seemed to be light losses caused by the object and hence there were probably errors which had to be accounted for.

The errors of the interferometer microscope have been discussed by Davies *et al.* [5, 6]. Because of the observation mentioned above, however, a special investigation was done in order to find out whether or not the results of this study were affected by the errors of the method.

Microphotographs of 10 erythrocytes were taken with the interference fringes at 9 different positions, keeping other factors such as focus and light conditions constant. In all, the fringes were moved a distance D , corre-

sponding to one wavelength optical retardation. At each setting of the fringes 10 photographs were taken and thus, for each cell, 90 determinations were made of the distance d . The mean of these determinations was regarded as the correct value and deviations from the mean were expressed as per cent of the distance D . For each cell, and for each setting of the fringes, the posi-

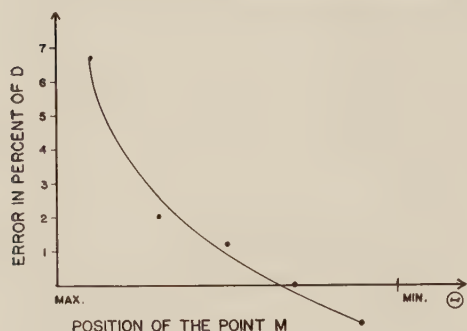


Fig. 7.—The mean error of the measurements, obtained as described under Error, is shown as a function of the position of the point M in Fig. 1.

tion of the point M in Fig. 1 was obtained from the known position of the cell, its mean d -value and the position of maxima and minima, which were calculated from equal density points on either side of the maximum and minimum in question. Deviations from the mean d -values were grouped according to the distance of the point M from a maximum ($0.05 \pm 0.05 D$, $0.15 \pm 0.05 D \dots 0.45 \pm 0.05$). Since it was found that the deviations had approximately the same numerical value but opposite signs at points situated symmetrically with regard to a maximum or a minimum, the signs of the deviations obtained as at position II in Fig. 1 were reversed. In this way, the deviations were collected in five groups, and the error, expressed as the mean of the deviations in each group, was related to the position of the point M as shown in Fig. 7. For objects situated as at position I in Fig. 1 the error is positive when the point M is situated near a maximum and negative when situated near a minimum. For objects situated as at position II the signs are reversed. In both cases the error is large near a maximum and small near a minimum.

Of the 21 cells represented in Fig. 6, 7 were situated as at position I in Fig. 1 and 14 as at position II. In the first case the last recorded values of d were therefore too high, and in the second case the initial values were too low. At the beginning of hemolysis, the velocity at which the recorded d/d_0 values decreased was then too high in 7 cases (when measured relative to the velocity at $d/d_0 = 0.55$) but too low in 14 cases. By taking into consideration the position of each cell and the error shown in Fig. 7, the magnitude of the

error of the mean relative velocity, v , was estimated, and it was found that the initial values of v were approximately 5 per cent too low. The deviation from the linear decrease of the velocity of hemolysis was therefore obtained in spite of the systematic error of the method.

CONCLUSION

From the definition of the permeability of the red cell membrane to hemoglobin (see eq. (7)), it follows that the derivate of the continuous curve shown in Fig. 6 is proportional to the permeability of the membrane and to the surface area through which the hemoglobin leaves the cell. Assuming that this surface area is constant during the fading of a red blood cell, it can be calculated from the slope of the continuous curve in Fig. 6 that the permeability is, at the beginning of hemolysis, more than 10 times as high as the permeability calculated for a free diffusion of hemoglobin (slope of the dotted line in Fig. 6) if hemolysis takes place within the same time in both cases. However, the fading time calculated for a free diffusion of hemoglobin across the entire surface of the cell [10], 0.16 seconds, corresponds fairly well to the shortest fading time observed [24], 0.20 seconds. This indicates that a permeability 10 times as high as that calculated under the original assumptions cannot exist unless there is an active mechanism, such as a pressure gradient across the membrane, which causes the hemoglobin to be actually ejaculated.

If it is instead assumed that the nonlinear decrease of the continuous curve in Fig. 6 is caused by a decrease of the surface area from which the hemoglobin is released, it can be calculated that the surface area from which the hemoglobin escapes decreases more than 95 per cent from the onset of hemolysis until 50 per cent of the hemoglobin is lost and that it is then constant. If such a decrease does take place, it is difficult to understand why it should suddenly stop in the middle of the fading of a cell, leaving a constant area from which the remaining hemoglobin can escape.

Of these two possible explanations to the observed hemolysis of red blood cells, it is believed that the mechanism involving an ejaculation of hemoglobin across a constant surface area is most probable. Consequently, there should exist a mechanically resistant membrane in order to explain the existence of a pressure gradient and the abrupt onset of hemolysis. It is also necessary that, if the high intracellular osmotic pressure is caused by a dissociation of hemoglobin aggregates into molecular hemoglobin, this should occur during the prolytic swelling of the cells or be such a rapid process that it does not limit the velocity of liberation of hemoglobin.

SUMMARY

The hemolysis of 19 human and 8 sheep red blood cells was recorded on cinematographic pictures through a Cooke-Dyson interferometer microscope. From measurements on the film the fading of each cell was followed quantitatively. Results are presented from experiments on osmotic hemolysis, delayed hemolysis in a hypotonic glucose solution, immune body hemolysis and hemolysis caused by a photodynamic active substance (rose bengal). In all cases the principal results were the same:

1. The hemolysis of a red blood cell starts abruptly.
2. During the hemolysis of a red blood cell there is a continuous decrease of the velocity at which the hemoglobin leaves the cell.
3. At the beginning of hemolysis, the velocity at which hemoglobin leaves a red blood cell shows a rapid, nonlinear decrease when plotted as a function of the intracellular concentration of hemoglobin. Possible explanations to this nonlinear decrease are discussed and it is concluded that hemolysis is best explained by the occurrence of a high intracellular osmotic pressure which causes the abrupt onset of hemolysis and ejaculation of the cell content.

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EFFECT OF CORTISONE ON LIVER NUCLEOPROTEINS¹

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PREVIOUS reports have shown that cortisone produces a decrease in concentration of nucleic acids in normal [2] and regenerating liver [5, 11]. This decrease in nucleic acid concentration has been attributed to an increase in fat and carbohydrate content of the liver [11] following cortisone administration. However, Bass and McArdle [2] have reported a decrease in average desoxyribose nucleic acid (DNA) content per nucleus, which they postulated might be explained by modifications in the polyploid distribution of liver nuclei. To pursue this hypothesis the present study was initiated.

The data presented here support the idea that the alteration in average DNA content per nucleus can be explained by shifts in the percentage of parenchymal nuclei in each ploidy class. However, modifications in nucleoprotein patterns are transient and are not sustained by continued cortisone administration.

MATERIALS AND METHODS

Male Swiss Albino mice (20-30 g) were used in this study. The experimental animals were fed Purina fox chow *ad libitum* and given free access to water at all times. Cortisone (cortone acetate, Merck) 100 μ g per gram of mouse per day was administered subcutaneously. Control animals were pair fed with the experimental group, had free access to water, and were injected with an equivalent amount of suspension medium.² Animals were killed by decapitation at the same time each day. The liver was removed, weighed, and samples taken for analysis. A 10 per cent homogenate of liver tissue was prepared by methods previously used in this laboratory [2]. The nucleic acids were extracted from aliquots of this homogenate and quantitatively determined by the method of Schneider [12]. Nitrogen was determined by the microkjeldahl procedure [9], fat by the method of Payne [10], and glycogen by the method described by Good *et al.* [6]. Pair fed control animals were sacrificed each day with the experimental animals. Since no significant alterations were observed due to dietary intake, these animals were considered together as a control group. The number of animals in each group is given in parenthesis in each table. Representative liver tissue for histological examination was fixed in 10 per cent formalin. The DNA content per individual nucleus was measured for 50 parenchymal

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² Cortisone acetate suspension medium was donated by Sharp and Dohme Division of Merck and Company.

nuclei in each of 5 mice per group by the method of Swift [13] and polyploid frequencies were determined by eye for at least 1000 hepatic nuclei per animal. Binucleate counts were made on tissues sectioned at 5 μ , nuclei stained by the Feulgen technique and the cytoplasm counterstained with fast green. The percentage of parenchymal nuclei included in the counts of total liver nuclei was estimated on 5 μ sections.

RESULTS

Cytoplasmic alterations.—A dose of cortisone which had been shown to produce a negative nitrogen balance in the liver was chosen. The effects of this dose of cortisone on the hepatic cellular constituents are shown in Table I. Within 24 hours, an engorgement of the liver cells by glycogen and a decrease in the concentration of ribose nucleic acid (RNA) and nitrogen were observed. A significant increase in fat was observed from 3 to 7 days after administra-

TABLE I. *Effect of cortisone on cellular constituents of mouse liver.*

g Liver per 100 g body wt.	Glycogen %	Fat %	RNA ^a mg/g	Nitrogen ^a mg/g	DNA/nucleus (mg $\times 10^{-9}$)
Control (54): 5.66 $\pm$ 0.137 ^b	4.52 $\pm$ 0.287	3.62 $\pm$ 0.274	41.01 $\pm$ 1.201	107.3 $\pm$ 2.29	9.46 $\pm$ 0.40
24 hr. (10): 8.33 $\pm$ 0.194*	11.27 $\pm$ 0.320*	5.43 $\pm$ 0.794	34.2 $\pm$ 3.796	97.36 $\pm$ 2.719*	10.48 $\pm$ 0.389*
48 hr. (10): 7.74 $\pm$ 0.510*	9.05 $\pm$ 0.446*	4.59 $\pm$ 0.382	36.15 $\pm$ 2.68	94.98 $\pm$ 1.071*	8.49 $\pm$ 0.465
72 hr. (10): 7.07 $\pm$ 0.712*	4.85 $\pm$ 1.105	6.57 $\pm$ 0.236*	35.35 $\pm$ 1.79*	91.1 $\pm$ 1.500*	8.08 $\pm$ 0.135*
5 days (5): 6.36 $\pm$ 0.295	5.31 $\pm$ 0.510	11.21 $\pm$ 1.094*	30.1 $\pm$ 1.700*	94.1 $\pm$ 2.694*	9.25 $\pm$ 0.205
7 days (3): 5.06 $\pm$ 0.680	5.32 $\pm$ 1.071	9.67 $\pm$ 2.378*	43.4 $\pm$ 0.960	109.4 $\pm$ 1.743	9.95 $\pm$ 0.501
10 days (3): 6.93 $\pm$ 0.133	3.71 $\pm$ 0.936	1.19 $\pm$ 0.754	40.9 $\pm$ 0.908	110.3 $\pm$ 2.02	11.4 $\pm$ 0.252*
14 days (3): 6.21 $\pm$ 0.591	1.26 $\pm$ 1.050	1.26 $\pm$ 0.924	42.4 $\pm$ 1.120	120.5 $\pm$ 4.547	10.3 $\pm$ 0.569

* Indicates *p* value of less than 0.01 as calculated by Fisher's Table of *t* when compared with the control value in each column.

^a Calculated as mg/g of glycogen free, fat free, dry tissue.

^b Standard error (S. E.) computed by standard deviation (S. D.) = $\Sigma x^2 - \frac{(\Sigma x)^2}{N-1}$; S. E. = $\frac{\text{S.D.}}{\sqrt{N}}$.

TABLE II. *Effect of cortisone on distribution of polyploid nuclei in mouse liver.*

(a) Results obtained on 50 parenchymal nuclei/section measured microspectrophotometrically;
 (b) results obtained on 1000 parenchymal nuclei/section DNA content estimated by eye.

	Diploid %	Tetraploid %	Octaploid %	16-ploid %
Control (5)				
(a)	24.4 $\pm$ 0.317	66.8 $\pm$ 1.786	8.8 $\pm$ 1.34	—
(b)	26.38 $\pm$ 1.77	68.44 $\pm$ 1.232	5.28 $\pm$ 1.188	—
24 hr. (5)				
(a)	10.0 $\pm$ 1.305*	77.2 $\pm$ 2.05*	12.0 $\pm$ 2.45	0.8
(b)	15.76 $\pm$ 1.399*	79.34 $\pm$ 2.31*	4.9 $\pm$ 0.953	—
48 hr. (5)				
(a)	38.0 $\pm$ 1.413*	53.2 $\pm$ 1.73*	8.4 $\pm$ 1.32	0.4
(b)	38.68 $\pm$ 1.795*	55.42 $\pm$ 1.45*	5.7 $\pm$ 1.11	—

* Indicates *p* value of less than 0.01 as calculated by Fisher's table of *t* when compared with the control value.

tion of cortisone. This increase in fat concentration did not occur until the glycogen concentration had returned to normal. Mice that received cortisone for 10–14 days frequently developed infections and were in poor physical condition when sacrificed; however, the cytoplasmic constituents had returned to approximately control levels.

Alterations in parenchymal cell nuclei.—Modifications in the distribution of polyploid nuclei in the liver of mice treated with cortisone are shown in Table II, and histograms from these animals can be seen in Fig. 1. Because all of the tissues represented in this figure were not sectioned, hydrolyzed and stained at the same time, minor shifts in peak values represent alterations due to technique. During the first 24 hours of treatment, there is a striking reduction in the per cent diploid nuclei observed; a reduction which is restored to normal and above normal values in the following 24 hour period. Although these rapid alterations in mean DNA per nucleus are small, they are statistically significant and the shifts in percentage of nuclei in each ploidy class appear to support these differences. A comparison of the magnitude of these alterations is seen in Table III. It is difficult to attach much significance to values obtained with mice which had been under cortisone treatment for from 10 to 14 days as the ploidy distribution was more variable. However, in general, these animals showed a higher percentage of larger nuclei.

The decrease in percentage of parenchymal cells containing diploid nuclei could be due either to selective destruction of nuclei, to fusion of nuclei in binucleate cells, or, less likely, to fusion of cells and subsequent fusion of the nuclei. No evidence of necrosis was noted in the sections examined. That fusion of nuclei occurred is supported by a reduction in the total number of

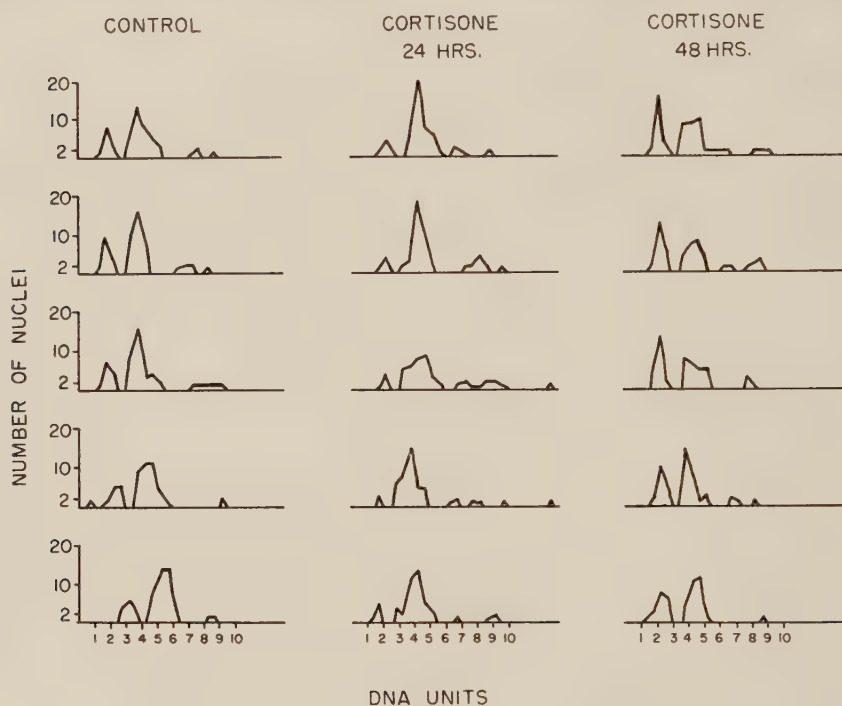


Fig. 1.—Polyploid distribution in mouse liver following cortisone administration.

TABLE III. *Mean DNA/nucleus computed by biochemical and photometric methods.*

Method of analysis	Control	24 hr.	Change %	48 hr.	Change %
Biochemical ($\text{mg} \times 10^{-9}$)	9.46 ± 0.40	10.48 ± 0.389	+11.0	8.49 ± 0.465	-10.25
Microspectrophotometric (relative DNA units)*	4.388 ± 0.1	4.884 ± 0.21	+11.36	3.912 ± 0.08	-10.83

* Value represents mean DNA units per parenchymal nucleus, as the percentage of parenchymal nuclei did not vary significantly after cortisone administration. (Control = 75.6 ± 1.02 %; 24 hr. = 74.2 ± 0.733 %; 48 hr. = 75.3 ± 0.526 %.)

nuclei per liver (5.45×10^8 to 4.26×10^8) as well as a decrease in the percentage of binucleate cells (26.3 to 16.4 per cent). The increase in the frequency of diploid nuclei at 48 hours might be due to an increase in mitotic index. Counts of number of dividing nuclei in parenchymal cells of control mice revealed a mitotic index of 0.02 ± 0.014 per cent. Twenty-four hours after cortisone no mitotic figures were observed, whereas forty-eight hours after cortisone there was a mitotic index of 0.004 ± 0.004 per cent.

DISCUSSION

Previous investigations [2, 5, 11] have shown a decrease in nucleic acid concentration in the liver following cortisone administration. The data presented here suggest that this action on nucleic acid concentration in the liver is transient. The concentrations of RNA, nitrogen, glycogen, and fat return to normal levels even though the animals continue to receive cortisone at the same dosage level. This indicates an adaptation of the metabolic processes of the liver to the effects of this agent. Using the periodic-acid-Schiff's reagent (PA-S) technique Williams *et al.* [14] have reported an increased deposition of glycogen after 7 days of cortisone administration; however, biochemical determinations reported in Table I do not show a significant alteration. Modifications in the distribution of polyploid nuclei in the liver offer an explanation for the alterations in the average DNA per nucleus. It is known that with age, the percentage of diploid nuclei in the liver decreases, and it has been postulated that starvation [3] or the presence of carcinoma [1] is accompanied by an increase in the percentage of nuclei of the higher ploidy classes. In the present work it was observed that during the first 24 hours the percentage of diploid cells dropped sharply. That this may be due to fusion of the nuclei in binucleate cells is suggested by the decrease in the percentage of binucleate cells and by the apparent loss of total nuclei from the liver. Although cortisone is known to be a mitotic inhibitor and mitotic indices reveal a drop in mitotic index at 24 hours, it is difficult to adequately explain the alterations reported in the ploidy distribution by an effect on the mitotic rate as the mitotic index in the liver of the adult animal is low [4]. However, since studies of the diurnal mitotic rhythm were not included in these experiments, it is possible that cortisone may produce more drastic alterations in the mitotic index during the peak of mitotic activity. Time studies involving the period from 24–48 hours after initiation of cortisone treatment might aid in elucidating the origin of these diploid nuclei.

The alteration of ploidy distribution appears to occur in three stages; an initial shift from the diploid to tetraploid nuclei which may be due to a fusion of the nuclei; a secondary rise in the number of smaller nuclei which continues throughout the active adaptation of the hepatic cell; and finally, a shift toward larger nuclei which accompanies the deteriorating physical status of the animal.

It appears that modifications of the polyploid distribution of nuclei in mouse liver are only temporary as are the alterations in the cytoplasmic constituents. However, in the mouse these cytoplasmic changes both appear and disappear more rapidly than has been reported in the rat [7]. It has been suggested recently by Lowe and Rand [8] that cortisone affects the degree of polymerization of RNA present in rat liver. However, our method of extraction would not allow this differentiation to be made.

SUMMARY

Administration of cortisone 100 $\mu\text{g/g}$ of mouse/day to male Swiss Albino mice produces only temporary modifications of the cellular components of the liver which indicate rapid adaptation of this tissue to cortisone. There is an increased deposition of glycogen during the first 24 hours of cortisone administration followed by a rapid return to normal values. Fat concentration in the liver cell reaches a peak at 5 days. Concentrations of RNA and nitrogen are lowered temporarily with this large dose of cortisone and return to control levels within 7 days. It seems reasonable to conclude that modifications in ploidy distribution of hepatic parenchymal nuclei induced by cortisone can explain the alterations in average DNA content per nucleus.

The technical assistance of Miss Catherine Cheatham is gratefully acknowledged.

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ÉTUDE AU MICROSCOPE ÉLECTRONIQUE D'UNE BACTÉRIE SULFUREUSE, *THIOVULUM MAJUS* HINZE

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Généralités, écologie

CERTAINES Bactéries sulfureuses incolores marines retiennent l'attention par quelques caractères assez exceptionnels, tels que leurs grandes dimensions, leur forme ovoïde et leur très rapide motion gyrotoire.

Hinze [4] a décrit sous le nom de *Thiovulum majus* et de *T. minus* deux de ces formes curieuses, et les a comparées à celle que Warming 1875 avait précédemment dénommée *Monas mülleri* en admettant son identité avec le *Volvox punctum* de O. F. Müller¹.

Thiovulum majus Hinze se développe abondamment dans les milieux marins littoraux riches en matières organiques dont la décomposition entraîne la production d'hydrogène sulfuré. Ce microorganisme, de forme ovoïde, mesure 11 à 18 μ suivant son grand axe, contre 9 à 17 μ suivant son diamètre transversal; le corps est plus ou moins chargé de globules de soufre très réfringents et anisotropes (Fig. 1 et 2); la présence de ces globules communique au *Thiovulum* un aspect blanc en lumière incidente, et noir en lumière transmise; bien que distribués dans toute la masse protoplasmique, leur accumulation est plus dense dans la région que l'on dira antérieure en se référant au sens du mouvement, la région postérieure étant plus riche en cytoplasme.

Le *Thiovulum* se multiplie abondamment dans les flaques littorales résiduelles au cours des périodes de morte eaux, et *in vitro*, au laboratoire, dans les récipients contenant avec de l'eau de mer très lentement renouvelée, un fond de sable vaseux ou des Algues en décomposition. Les populations très denses qui se forment dans ces conditions se rassemblent, si le milieu liquide reste calme, en nuages blancs et plus souvent en voiles ténus dont les surfaces courbes se déforment, se déplacent, se compliquent ou s'élargissent lentement.

On provoque aisément la formation de tels amas en immergeant dans une riche suspension de *Thiovulum* de petits segments de tubes capillaires pré-

¹ On trouvera dans le mémoire de Hinze 1913 la documentation historique et bibliographique.

ablement remplis de gélose contenant du sulfhydrate d'ammonium très fortement dilué. Les Sulfobactéries s'accumulent rapidement à chaque extrémité du tube (Fig. 4) en un nuage très dense, mais temporaire, car bientôt il s'écarte et s'élargit en voiles courbes ou en masses vésiculeuses, complexes et irrégulières qui se déplacent vers la surface libre du liquide (Fig. 5). Quelques expériences simples montrent facilement que la position des *Thiovulum* marquée par les voiles en déplacement dépend des concentrations relatives, dans le milieu, de H_2S qui diffuse à partir du fond et de O_2 qui diffuse à partir de l'air; c'est à dire des vitesses relatives (1) de la diffusion de ces corps dans l'eau de mer et (2) de leur utilisation par la Sulfobactérie. Mais d'autres conditions interviennent d'autre part; le libre parcours des *Thiovulum* est limité par la probabilité des rencontres individuelles qui dépend elle même de la vitesse propre de ces Bactéries et de la densité de leur population. Comme on a pu l'observer avec d'autres microorganismes nageurs ces facteurs sont responsables de la formation, à l'intérieur même d'un voile en mouvement, de condensations localisées dessinant un réseau plus ou moins régulier¹.

TECHNIQUE

Le matériel utilisé au cours de ce travail a été récolté, examiné ou préparé à Concarneau et à Roscoff avec la collaboration expérimentée de M^{lle} Michelle Gauchery, à qui nous adressons ici tous nos remerciements.

Il est remarquable que les nuages de *Thiovulum* manifestent une certaine cohésion bien que chacun des microorganismes constituant les nuages reste en continuelle agitation et en rotation très rapide. Les réactifs fixateurs semblent renforcer cette cohésion, comme si elle était dûe à l'existence d'une gelée interstitielle précipitable². Ce simple fait facilite grandement les manipulations et permet, au prix de quelques précautions, de traiter les petits amas de *Thiovulum* comme des fragments de tissus au cours de la fixation et des premiers lavages.

Les examens au microscope électronique [10] ont été effectuées sur des amas de *Thiovulum* fixés au mélange chromo-osmique additionné de NaCl pour éviter le déséquilibre osmotique avec l'eau de mer, et tamponné à pH 8. Après lavage, les amas étaient d'abord inclus dans la gélose pour éviter leur dispersion. Les blocs, très petits, étaient ensuite déshydratés puis inclus au méthacrylate de butyle; les sections ultrafines, effectuées avec le microtome Porter Blum, ont été examinées au microscope RCA type EMU 3B.

¹ J. B. Loefer et R. B. Melferd 1952 ont étudié quelques cas de « pattern formation by free-swimming microorganisms ». (*Am. Naturalist* **86**, 325 (1952).)

² Un fait analogue a été constaté avec les populations denses de deux *Amphidinium* psammobies marins; ces Péridiniens produisent un mucilage formant dans l'eau de mer une gelée très hydratée qui n'empêche pas les mouvements des *Amphidinium* bien qu'elle puisse accuser une certaine tenacité.

Les préparations utilisées pour les examens photomicroscopiques ont surtout comporté des frottis; ceux-ci étaient effectués soit avec du matériel préalablement fixé sous forme dispersée, puis lavé et rassemblé par centrifugation ou par décantation avant d'être étalé sur lame porte-objet avec de l'albumine glycinée que l'on coagule à l'alcool formolé; soit en desséchant le matériel vivant sur lame porte objet et en opérant une post-fixation au liquide de Schaudinn, ou encore à la solution iodoiodurée de Lugol etc. etc. Les diverses colorations appliquées à ces frottis avaient pour objet la caractérisation éventuelle de mitochondries, de structures nucléaires, et d'autres structures cytologiques¹.

Structures cytoplasmiques

Membrane d'enveloppe.—La réaction de McManus–Hotchkiss (PAS) colore légèrement mais nettement la surface des *Thiovulum*, indiquant ainsi l'existence probable d'un revêtement polysaccharidique. Les micrographies électroniques montrent ce revêtement sous l'aspect d'un trait continu dont l'épaisseur est inférieure à 50 Å et qui enveloppe toute la masse protoplasmique dont elle paraît être séparée par un espace étroit. Chez les individus en voie de cytolysé, le cytoplasme se fragmente en une série de sphérules granuleuses de dimensions très diverses, mais celles-ci restent incluses, sous la fine membrane périphérique persistante (Fig. 9). Chez les individus normaux la masse cellulaire apparaît limitée au dessous de la membrane périphérique par une membrane cytoplasmique plus dense dont il sera question plus loin.

Cytoplasma.—L'examen du cytoplasma chez le *Thiovulum* vivant est rendu difficile par l'extrême mobilité du microorganisme et par la présence des inclusions réfringentes de soufre; après fixation et dissolution de celles-ci, il apparaît comme une masse alvéolaire un peu plus dense à l'un des pôles. Les sections ultrafines examinées au microscope électronique montrent entre les vacuoles correspondant aux inclusions de soufre, une masse très finement granuleuse, irrégulièrement cloisonnée par un système complexe de doubles membranes sur lesquelles on reviendra plus loin. L'aspect finement granuleux traduit, probablement, et tout à la fois, la précipitation d'un colloïde protéique, et la présence des « 100-200 Å granules » décrits chez diverses Bactéries (voir [2]).

L'examen au microscope électronique de quelques milliers de sections de *Thiovulum* n'a jamais montré aucune structure qui puisse suggérer la présence de mitochondries. De même les colorations électives pour le chondriome

¹ Les premiers résultats de ces examens ont été exposés par l'un de nous aux journées cytoembryologiques belgo-néerlandaises de Louvain en 1952. Le résumé intitulé « Caractères cytologiques d'une Sulfobactérie, *Thiovulum majus* Hinze », inclus dans le programme roneotypé de cette réunion, n'a pas été publié ultérieurement.

ne mettent en évidence à l'examen photomicroscopique, aucun élément granulaire ou filamenteux, en dehors toutefois de l'organite polaire dont les caractères particuliers et l'ultrastructure seront décrits plus loin.

Les inclusions de Soufre.—Les inclusions cytoplasmiques réfringentes caractéristiques des *Thiovulum* à l'état de vie active ont une forme globuleuse ou ovoïde; mais, bien que limitées par des surfaces courbes, leur forte biré-

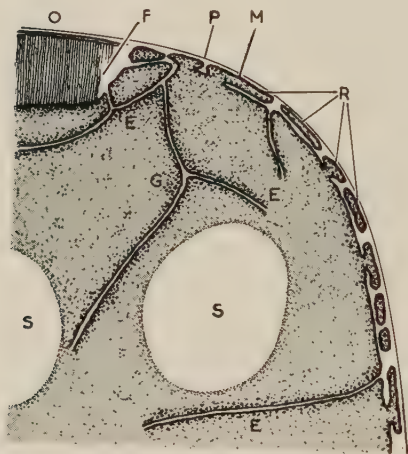


Fig. A.—Structure fine de *Thiovulum*; représentation schématique d'une section tracée d'après plusieurs micrographies électroniques. *P*, pellicule externe; *M*, membrane cytoplasmique; *R*, réseau superficiel; *E*, cytomembranes; *G*, granules « 100–200 Å »; *S*, espace correspondant aux globules de soufre; *O*, organite antapical et *F*, ses fibrilles parallèles.

fringence en lumière polarisée atteste leur texture cristalline (Fig. 2). Faiblement solubles dans les alcools, ces inclusions se dissolvent rapidement et totalement dans le xylol ou le benzène, laissant dans le cytoplasme autant de vacuoles que les micrographies électroniques montrent limitées par un trait fin et continu, sorte de membrane interfaciale d'épaisseur infime (Fig. A dans le texte, et Fig. 8, 10, 14).

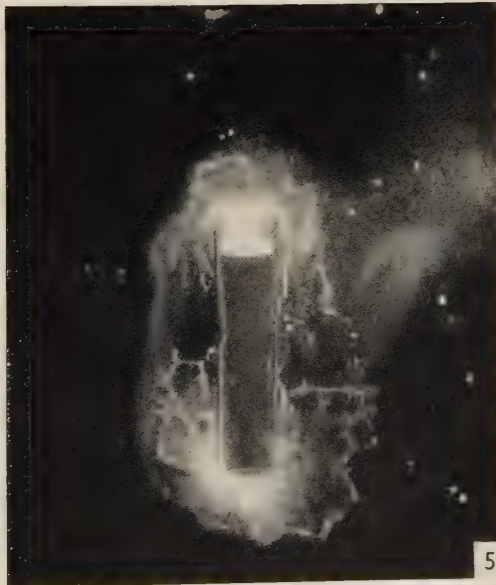
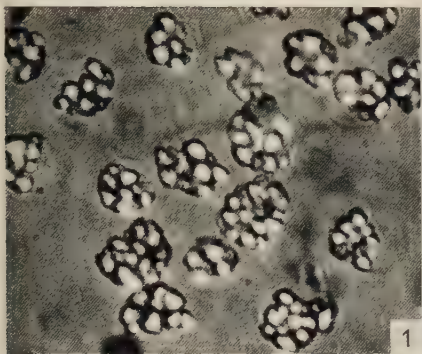
L'évaporation sur lame porte objet d'une goutte de la solution benzenique donne des cristaux de soufre (Fig. 3).

Fig. 1 et 2.—Un même groupe de *Thiovulum* séché, monté au sirop d'Apathy, photographié en lumière ordinaire (1) et en lumière polarisée (2) pour montrer les globules de soufre réfringents et anisotropes.

Fig. 3.—Cristaux de soufre obtenus par évaporation de l'extrait benzenique de *Thiovulum*; lumière polarisée.

Fig. 4.—Accumulation des *Thiovulum* aux deux extrémités d'un tube capillaire contenant de la gélose riche en H_2S . ($\times 4$ environ; lumière réfléchie).

Fig. 5.—Le même tube quelques minutes plus tard montrant la formation des voiles ($\times 4$ environ, lumière réfléchie).



Zone ectoplasmique.—Les sections tangentes à la surface du corps montrent un aspect curieusement réticulé dû à la juxtaposition de plages en reliefs dont les contours irréguliers sont aussi divers que les dimensions (Fig. 17). Les sections perpendiculaires à la surface indiquent aussitôt que ces reliefs correspondent à l'étalement de plissements protoplasmiques superficiels développés au-dessous de la fine membrane enveloppante, et formant une zone corticale mince extrêmement découpée (Fig. A dans le texte, et Fig. 10, 11, 13). Il apparaît ainsi que le cytoplasme superficiel se soulève et s'étale en diverticules pédiculés prenant souvent sur les coupes l'aspect d'îlots plats, totalement isolés au-dessus de la surface du corps, et dont le diamètre peut atteindre 100 à 500 m μ ; la pellicule cytoplasmique qui entoure ces diverticules s'étale de ce fait sur une surface considérable.

Doubles membranes intracytoplasmiques.—En même temps que la pellicule s'étale sur une série d'évaginations cytoplasmiques superficielles, elle augmente encore sa surface en s'invaginant sous forme de plissements étroits et profonds; ces plissements apparaissent alors comme autant de doubles membranes qui s'enfoncent dans l'endoplasme sous diverses incidences, obliques, secantes ou radiales (Fig. A et Fig. 10, 11, 12, 13, 14).

Les doubles membranes comportent, de part et d'autre d'un espace étroit de 100 à 200 Å deux couches denses, absorbantes, dont les faces en regard (c'est-à-dire externes) paraissent à peu près lisses tandis que les faces internes, au contact du protoplasma, sont parsemées de granules ou d'amas granuleux et paraissent irrégulières de ce fait. Leur épaisseur propre peut être de l'ordre de 50 Å.

En profondeur, dans les masses cytoplasmiques qui séparent les globules de soufre, les doubles membranes s'étendent en cloisons incomplètes, souvent contournées, recourbées ou ramifiées en un réseau alvéolaire.

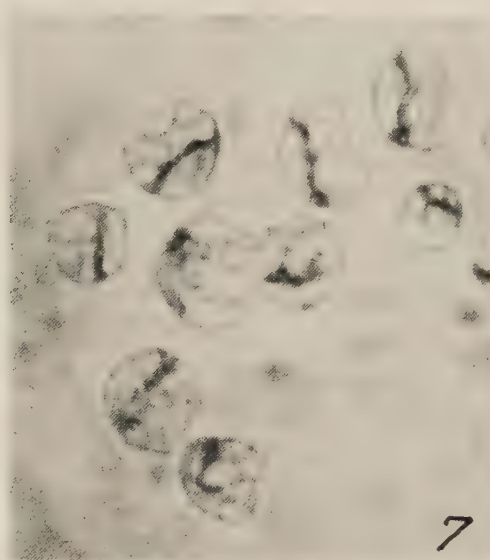
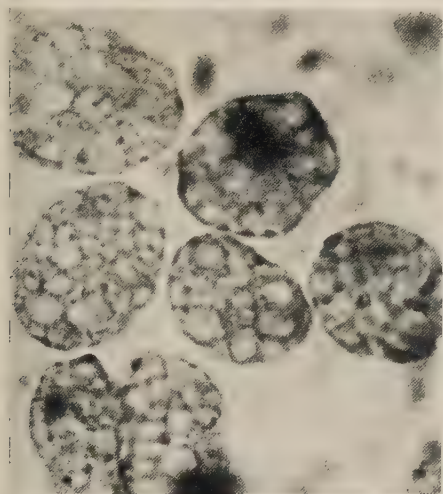
En première approximation on peut considérer les doubles membranes intracytoplasmiques du *Thiovulum* comme homologues aux structures désignées par Sjöstrand [11, 12, 14] sous le nom de « cytomembranes ».

Inclusions basophiles.—La double coloration au bleu de toluidine-éosine montre à l'examen photomicroscopique, des granules de moins de 1 μ ,

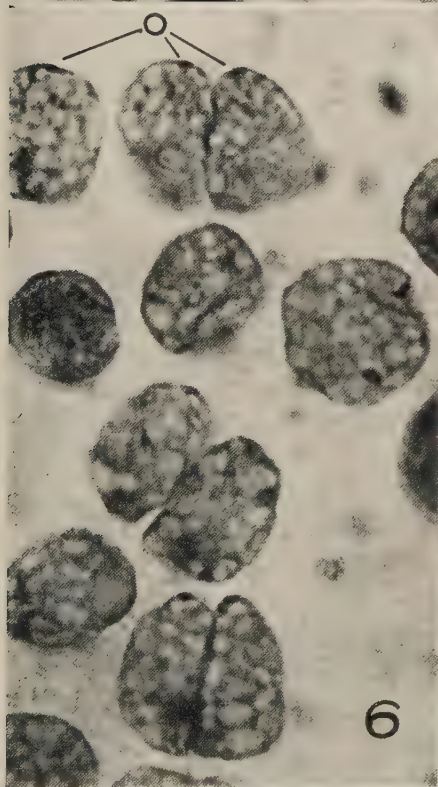
Fig. 6.—*Thiovulum* colorés à la fuchsine acide et au bleu d'aniline après fixation au sublimé acétique montrant l'organite antapical, O.

Fig. 7.—Réaction de Feulgen montrant la bande nucléaire N.

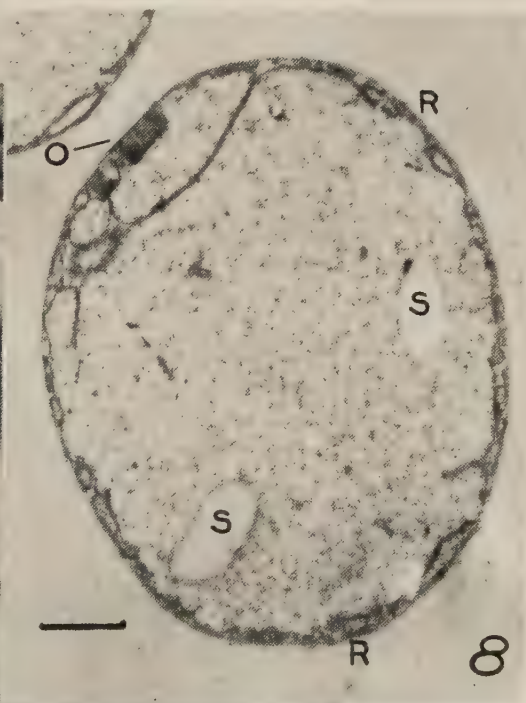
Fig. 8.—Micrographie électronique d'une section passant par l'organite antapical O; R, réseau superficiel; S, espaces correspondant aux inclusions de soufre. *Nota:* le trait porté sur les micrographies électroniques représente 1 μ , ou (avec indication chiffrée) 0.2 μ .



7



6



R

S

S



R

8

colorés en bleu sombre (surtout après mordantage chromique), dispersés entre les vacuoles correspondant aux grains de soufre; l'hématoxyline Delafield donne une image comparable. L'hydrolyse chlorhydrique supprime la colorabilité de ces granules basophiles, lesquels pourraient être de nature ribonucléoprotéique.

Les sections ultrafines examinées au microscope électronique ne montrent pas, à première vue, de corpuscules comparables à ces granulations basophiles, à moins toutefois, que celles-ci ne correspondent aux amas granuleux accolés aux « cytomembranes »; on sait en effet (voir [2]) que les « 100–200 Å granules » connus chez diverses Bactéries contiennent de l'acide ribonucléique.

Le corpuscule antapical

Les colorations à la fuchsine acide après fixation chromo-osmique ne montrent, comme il a été dit, aucune granulation d'aspect mitochondrial, mais elles colorent très électivement un corpuscule superficiel, simple ou double, situé au pôle cytoplasmique du *Thiovulum*. On notera aussitôt, cependant, que la fuschsinophilie intense de ce corpuscule se manifeste avec les techniques les plus diverses et les moins adaptées à la coloration du chondriome (Fig. 6).

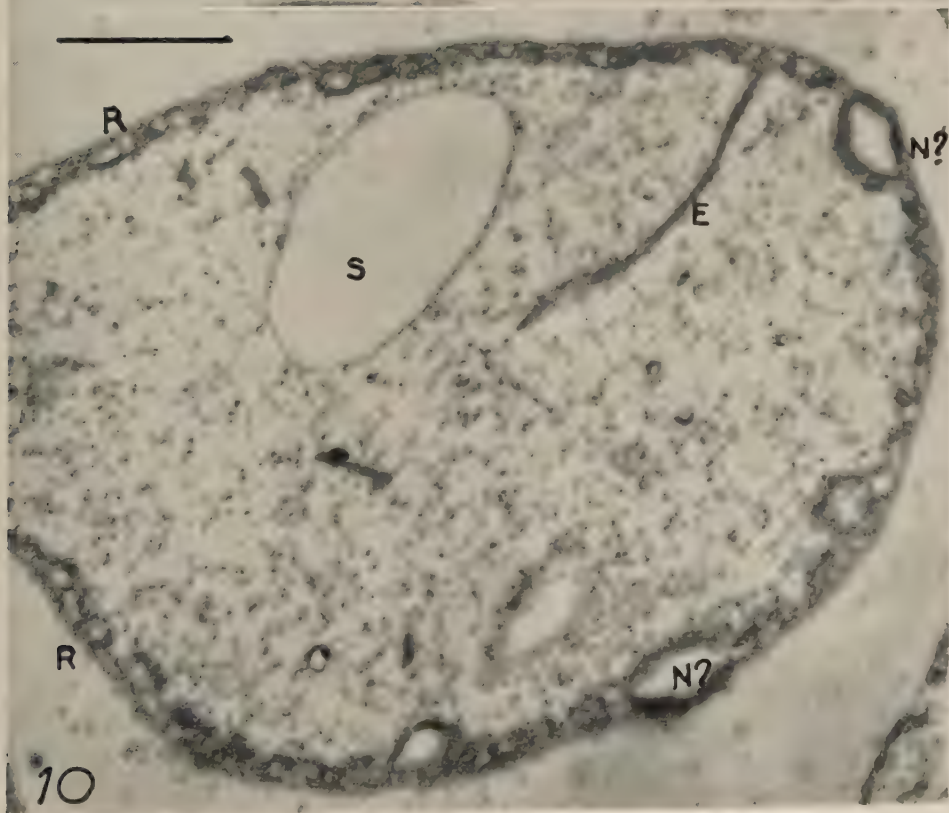
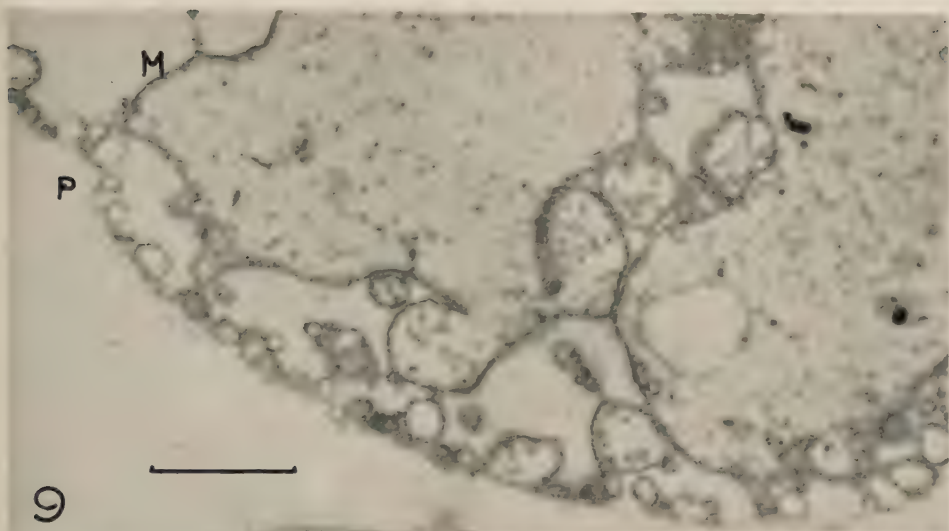
En dehors de son affinité très élective pour la fuchsine acide, le corpuscule antapical ne fixe pas les colorants basiques et ne retient pas l'hématoxyline ferrique.

Ce corpuscule polaire est un organite constant, permanent et probablement autoreproductible; il apparaît au microscope optique comme un corps superficiel, nettement délimité, de forme circulaire ou elliptique, mesurant près de 1 μ de diamètre (Fig. 6). Il apparaît double chez nombre d'individus, et cet état, qui semble être l'effet d'une division, précède la bipartition longitudinale du *Thiovulum* (Fig. 13, 14).

Les micrographies électroniques de sections coupant le corpuscule polaire sous des angles divers montrent celui-ci comme une plaquette discoïde large de 0,7 à 1 μ , épaisse de 0,3 μ environ, dont la structure fine évoque l'image d'une bordure en brosse qui serait incluse au-dessous de la mem-

Fig. 9.—Région superficielle d'un individu en cytolyse montrant, au-dessous de la pellicule externe *P*, non altérée, le cytoplasme fragmenté en gouttelettes sphériques limitées par une membrane fine, *M*.

Fig. 10.—Section montrant la masse cytoplasmique finement granuleuse, le réseau superficiel *R*, quelques doubles membranes endoplasmiques *E*, la place d'un grain de soufre *S*; deux espaces *N*? dans le réseau superficiel correspondant peut être à la bande nucléaire.



brane cellulaire (Fig. A et Fig. 8, 11, 12). La plaquette discoïde est en effet constituée par un large faisceau de courtes fibrilles, parallèles les unes aux autres et perpendiculaires à la surface du corps; la longueur de ces fibrilles correspond à la hauteur du corpuscule polaire, soit près de $0,3 \mu$; leur diamètre est de l'ordre de $100 \text{ \AA}$; elles présentent souvent un aspect moniliforme, comme si elles étaient constituées par une file de granules. Les sections transversales (Fig. 13) montrent que ces fibrilles sont assez régulièrement distribuées en quinconces; les espaces qui les séparent sont du même ordre de grandeur que leur propre diamètre.

Du côté périphérique les fibrilles se terminent en étroit contact avec la face interne de la membrane externe; mais celle-ci semble parfois dédoublée, comme si une pellicule spéciale unissait les extrémités périphériques de toutes les fibrilles.

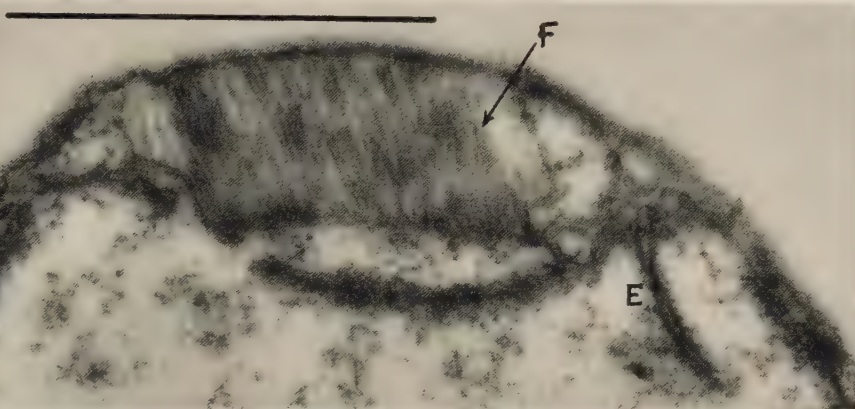
Du côté central ces mêmes fibrilles se terminent toutes sur un même niveau plan ou légèrement incurvé, marqué comme une sorte de membrane basale assez dense.

Latéralement, la membrane cytoplasmique précédemment décrite s'infléchit progressivement, à partir de la zone des îlots protoplasmiques superficiels, et vient rejoindre la base de la plaquette antapicale (Fig. 12) sous laquelle elle constitue sans doute la membrane basale. Le corps fibrillaire est donc enchassé dans une sorte de fossette protoplasmique dont la paroi s'invagine de place en place pour donner les doubles membranes cytoplasmiques.

La plaquette antapicale apparaît d'après l'ensemble de ces faits, comme un organe bien défini mais de nature énigmatique, car il ne semble comparable à aucune des ultrastructures cellulaires actuellement connues. Sa structure fine évoque sans doute celle d'un certain nombre de fibres protéiques à texture fibrillaire observées chez différents Protistes, dont les unes sont des éléments squelettiques (fibres et baguettes pharyngiennes des Ciliés p. ex.), et les autres des organites contractiles (myonèmes ectoplasmiques des *Stentor* et des *Spirostomum*, axostyle de *Pyrsonympha*); mais entre autres différences, on remarquera que les fibrilles de l'organe polaire du *Thiovulum* sont notablement plus fines et plus serrées que celles dont il vient d'être question.

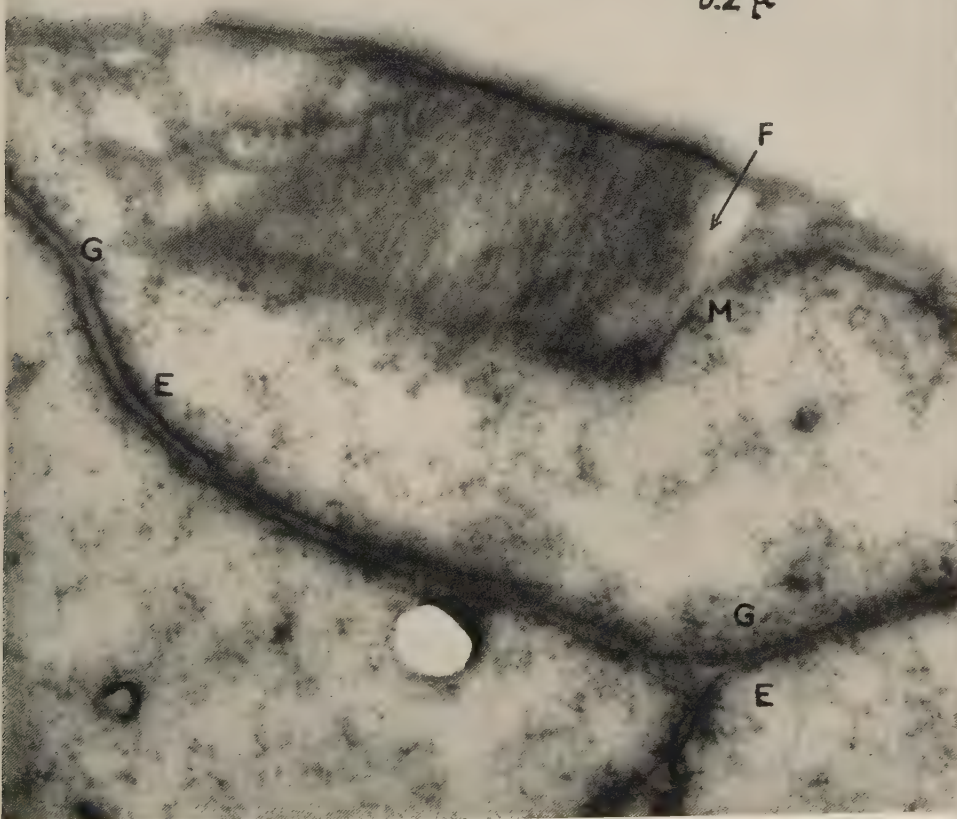
Fig. 11 et 12.—Deux sections à travers l'organe antapical montrant la masse des fibrilles parallèles *F*, l'invagination de la membrane cytoplasmique *M* et le réseau des doubles membranes endoplasmiques *E* bordées par les granules *G*.

11



12

0.2 μ



Noyau

La réaction de Feulgen met en évidence chez *Thiovulum* une bande superficielle, de forme irrégulière, allongée latéralement entre la zone antérieure la plus riche en inclusions de Soufre et le pôle cytoplasmique postérieur. Cette bande apparemment très mince, est faiblement mais nettement colorée en rose violacé (Fig. 7). La durée de l'hydrolyse chlorhydrique est importante avec une structure aussi ténue, et les meilleurs résultats sont obtenus après 3 à 4 minutes à 60°; la réaction faiblit très sensiblement après 8 minutes. Dans les meilleures conditions il est utile d'accentuer le contraste à l'aide d'un filtre vert pour étudier et photographier les préparations.

La même bande est également colorée, nettement et faiblement, par le bleu de toluidine agissant après qu'une hydrolyse chlorhydrique de 8 à 10 minutes a supprimé la basophilie du cytoplasme; l'aspect obtenu est exactement comparable à celui donné par la réaction de Feulgen.

Nous admettons que la bande basophile et Feulgen positive du *Thiovulum* représente le noyau de ce microorganisme; mais, à cet égard, l'examen des micrographies électroniques est assez décevant car il ne montre aucun aspect évoquant une texture nucléaire. Cependant, les micrographies à faible grossissement, d'intérêt topographique, montrent parfois, dans la zone périphérique occupée par les îlots cytoplasmiques superficiels, la présence d'une enclave ou de deux enclaves symétriques (Fig. 10, N^o), plus larges que les autres, d'aspect clair, de section elliptique, limitées par un contour sombre, qui pourraient être les sections transversales d'une bande nucléaire superficielle dont le matériel chromatinien (ou ADN-protéique) serait localisé sur la paroi.

Division

La division du *Thiovulum* est longitudinale; il semble qu'elle débute au pôle antérieur, riche en enclaves de soufre; la bande nucléaire, sub-équatoriale, accuse bientôt une double courbure, puis le sillon de bipartition s'accentue et sépare enfin les deux pôles cytoplasmiques qui emportent chacun un corpuscule fuchsinophile.

Les micrographies électroniques montrent au niveau du sillon de bipartition le développement des « cytomembranes »; celles-ci s'accroissent et se multiplient en un réseau endoplasmique bilamellaire qui isole une double série d'îlots cytoplasmiques disposés de part et d'autre du plan de division (Fig. 14, 15, 16).

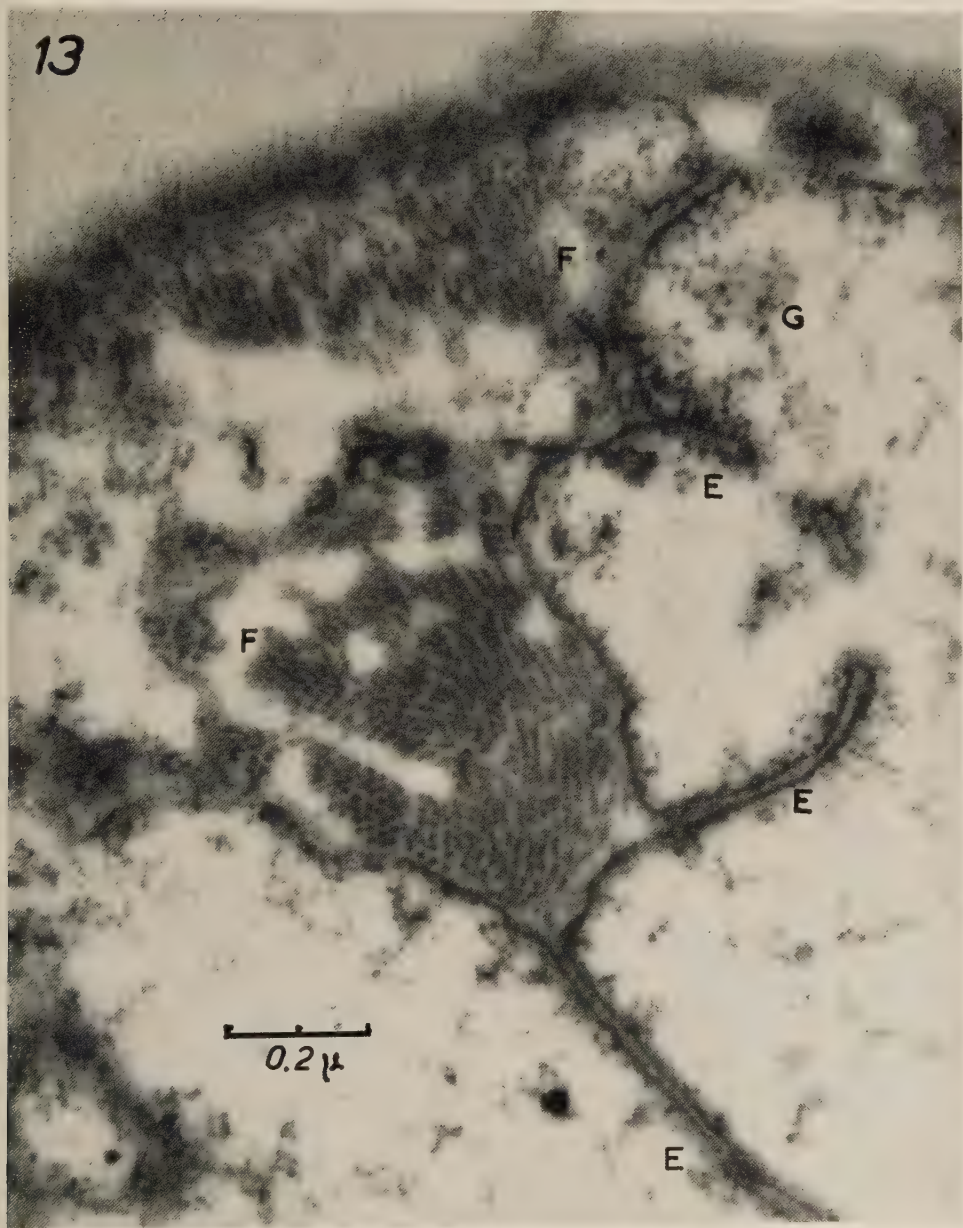


Fig. 13.—Section parallèle au plan d'un organite antapical, montrant ses fibrilles *F* coupées transversalement, la membrane cytoplasmique bordante et les doubles membranes endoplasmiques *E* avec les granules *G*. Le second organite antapical indiqué par une flèche est sectionné obliquement.

DISCUSSION

Caractères généraux.—Les dimensions du *Thiovulum*, sa forme ovoïde, la présence d'un corpuscule apical, sa division longitudinale, ses mouvements rapides évoquent à première vue, comme Hinze [4] le remarque à propos du *Monas mülleri*, l'idée d'un Flagellé. Cependant le métabolisme de ce microorganisme qui oxyde H_2S et accumule du soufre, comme l'absence de chondriome, sont autant de caractères bactériens. Il est intéressant, de ce fait, de revenir rapidement sur les structures cytoplasmiques et nucléaires du *Thiovulum* pour les comparer à ce que l'on connaît chez les Bactéries; mais il convient, au préalable de poser un difficile problème, celui de la motilité de cette Sulfobactérie très particulière.

Mouvement et organe propulseur.—Comme il a été dit plus haut les mouvements de translation du *Thiovulum* s'effectuent le pôle granuleux en avant, comme on peut le vérifier lorsque le mouvement gyrotoire se ralentit au début de la cytolyse. Hinze [4] croit pouvoir conclure du mouvement à la présence de flagelles locomoteurs qu'il cherche à mettre en évidence avec les techniques cytologiques adéquates. Les résultats obtenus semblent inconstants et incertains; après fixation au liquide de Flemming fort et coloration à la fuchsine concentrée il obtient l'image d'une ciliature péritriche; sa figure 21 est schématique et manque de précision.

En fait nous n'avons jamais réussi à mettre en évidence, chez *Thiovulum*, aucune apparence de flagelle, ni après colorations, ni après l'examen à l'état vivant en éclairage direct, en contraste de phase ou en fond noir. De plus, les très nombreuses micrographies électroniques examinées ne nous ont jamais montré aucun détail de structure que l'on puisse rapporter à la présence, ou seulement même au point d'insertion d'un ou de plusieurs flagelles du type bactérien.

Bien qu'un argument négatif soit peu satisfaisant, on peut se demander si *Thiovulum* ne serait pas en fait dépourvu de flagelle; mais s'il en était ainsi, la question se poserait aussitôt de savoir quel est l'agent ou l'organe moteur de ce microorganisme.

Structure du Thiovulum et cytologie bactérienne.—En comparant la structure du *Thiovulum* aux structures et aux ultrastructures actuellement connues chez les Bactéries, on ne doit pas oublier que la masse cytoplasmique de notre Thiobactérie est relativement considérable, même en tenant compte de l'espace occupé par les inclusions de soufre.

L'existence de corps basophiles a été signalée dans le cytoplasma du *Thiovulum* par Hinze qui les assimile à des grains de vultine. Ce sont

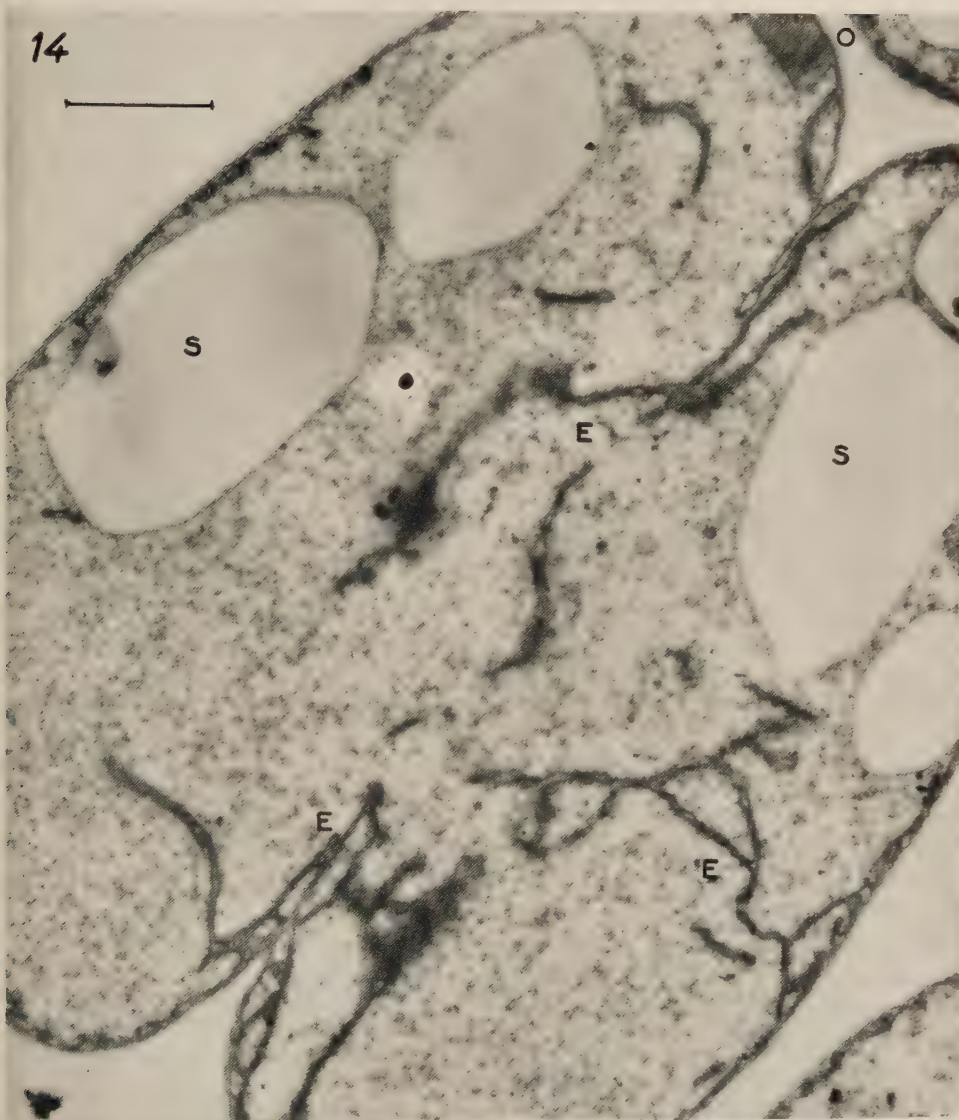


Fig. 14.—Stade de division montrant l'organe antapical *O* de l'un des deux individus; le sillon longitudinal; le réseau des doubles membranes *E*, et la place des grains de soufre *S*.

probablement les mêmes que nous avons mis en évidence par le bleu de toluidine après hydrolyse chlorhydrique. La question se pose de savoir à quelles structures fines correspondent ces granules de forme et de dimension

apparemment variables, et qui sont peut-être constitués par des ribonucléoprotéines¹.

On a vu que le cytoplasma du *Thiovulum* renferme de nombreux corpuscules denses que l'on pourrait assimiler aux « 100-200 Å granules » comme chez nombre de Bactéries (voir [2]); or ces granules contiennent de l'acide ribonucléique comme les grains ergastoplasmiques de Palade [8], et, comme ceux-ci, se trouvent accolés aux cytomembranes. Les cytomembranes prennent de ce fait les caractères d'un « endoplasmic reticulum » au sens de Porter et Palade [7, 9], ou de doubles lamelles ergastoplasmiques (voir Bernhard et collaborateurs [1]).

Ces cytomembranes granuleuses ne semblent pas avoir été décrites chez les Bactéries (voir [2]) et leur développement chez *Thiovulum* paraît être en rapport avec l'importance relative de la masse cytoplasmique².

Il est vraisemblable que les grains basophiles observés au microscope optique correspondent à des agrégats de granules, ou bien encore à quelques points où les « cytomembranes » forment par leurs ramifications et leurs plissements des amas particulièrement denses.

L'absence de toute espèce de structure que l'on puisse comparer à des mitochondries est entièrement vérifiée par les micrographies électroniques; malgré nombre de discussions sur lesquelles il est inutile de revenir ici (le lecteur trouvera toute la documentation dans les mises au point de Bradfield [2], Marshak [5], Mudd [6]); ce fait est en plein accord avec les données actuelles sur la cytologie bactérienne.

La bande nucléaire mise en évidence chez *Thiovulum* par la réaction de Feulgen ou par le bleu de toluidine présente des caractères structuraux trop incertains, même au cours de la division, pour justifier une comparaison intéressante avec les noyaux bactériens en général.

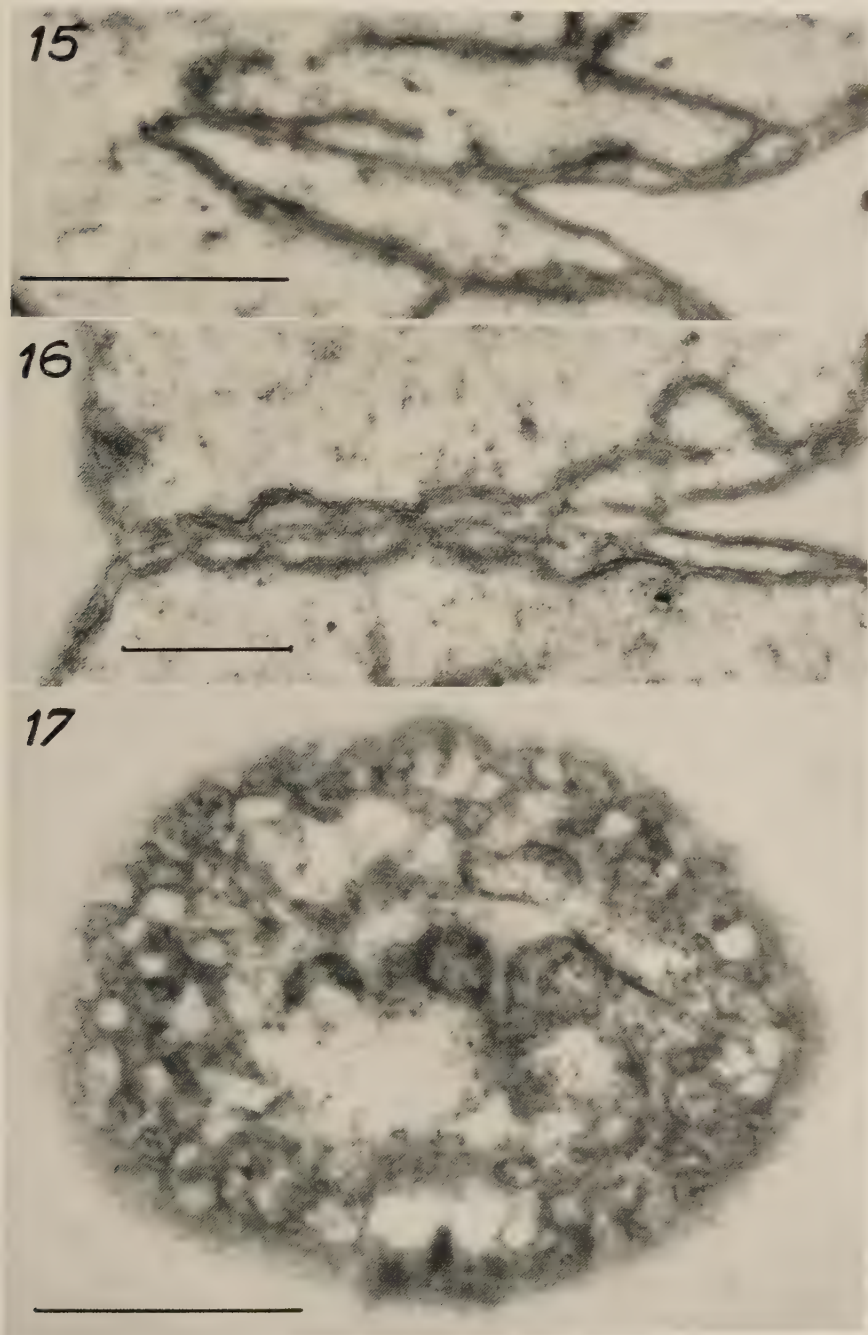
L'organite antapical. — On sait que le corpuscule antapical fuchsophile du *Thiovulum* ne semble correspondre à aucun organite cellulaire déjà connu; en l'absence, chez ce microorganisme, de tout élément de nature flagellaire, il est permis d'imaginer qu'il puisse jouer le rôle d'organite moteur. Cette hypothèse ne repose sur aucune donnée positive, et ne peut être complétée que par des suggestions non moins hypothétiques. On pour-

¹ Nous n'avons pas décelé chez *Thiovulum* la présence de polyphosphate (voir [3]) et la coloration par le bleu de Toluidine est orthochromatique.

² Chez *Bacillus thuringiensis* (voir [2]) une micrographie montre l'apparence d'une double membrane.

Fig. 15 et 16. — Deux aspects du sillon longitudinal montrant le développement des doubles membranes endoplasmiques traçant le plan de division.

Fig. 17. — Section tangente à la périphérie du corps montrant le réseau superficiel.



rait ainsi supposer que le dispositif fibrillaire est contractile et que le corpuscule antapical fonctionne comme un organe pulsant; ou bien encore que les échanges liés à l'oxydation de H_2S provoquent à son niveau un flux de substance capable de provoquer un mouvement par réaction. Il reste bien entendu que ces suppositions n'ont aucune base objective.

RÉSUMÉ

Thiovulum majus Hinze est une Bactérie sulfureuse marine très mobile dont la grande taille s'accompagne d'intéressantes particularités cytologiques, révélées ou précisées par la microscopie électronique.

Le noyau apparaît comme une bandelette périphérique Feulgen-positive sub-équatoriale.

Le cytoplasme ne contient pas d'éléments structurés que l'on puisse identifier à des mitochondries.

Par contre un important ensemble de « cytomembranes » ou doubles membranes, dont le contour est marqué par l'accumulation de granules intracytoplasmiques se ramifie dans les travées protoplasmiques entre les inclusions de soufre et semble correspondre à un ergastoplasme basophile (« endoplasmic reticulum »).

Bien que *Thiovulum* se meuve avec rapidité, aucun flagelle n'a pu être mis en évidence.

Le pôle postérieur (ainsi défini d'après le sens du mouvement) porte un organite à structure fibrillaire, de nature énigmatique. La possibilité d'un rôle moteur est envisagée.

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STRUCTURE FINE D'UN FLAGELLÉ CHRYSOMONADIEN :
CHROMULINA PSAMMOBIA

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Chromulina psammobia Fauré-Fremiet [12] est un Flagellé chrysomonadien marin dont les populations naturelles, de forte densité, apparaissent à marée basse comme des taches de couleur jaune verdâtre à la surface des sables humides exondés à basse mer; on le trouve en abondance sur des plages de sable fin à faible déclivité, retenues et protégées par des groupes rocheux¹; il s'accumule plus particulièrement le long de petits filets de ruissellement. La récolte de très nombreux individus s'effectue aisément en prélevant avec une lame de matière plastique la couche superficielle de sable coloré. Au laboratoire, ce sable est distribué dans des cuvettes plates et recouvert d'une faible couche d'eau de mer. Le rythme de marée [12] se poursuit assez régulièrement *in vitro* pendant quelques jours; l'agglutination des *Chromulina* (au stade lucifuge correspondant à la période de submersion) fournit un matériel facile à manipuler au cours des diverses opérations.

On a utilisé comme fixateur un mélange chromo-osmique salé dont la concentration est proche de celle de l'eau de mer, ramené aux environs de pH8 avec le tampon acétate de Na-Veronal selon Palade, et contenant un peu de MgCl₂ et de SO₄Na₂ pour équilibrer l'effet gonflant de NaCl; il était obtenu en mélangeant à parties égales les deux solutions suivantes :

OsO ₄ à 2 % (tamponné)		1 vol.
Cr ₂ O ₇ K ₂	2	} 1 vol.
ClNa	5	
MgCl ₂	1	
SO ₄ Na ₂	0,8	
eau distillée	100	

L'inclusion était faite au méthacrylate de butyle.

Les sections effectuées avec le microtome Porter-Blum ont été examinées au microscope électronique R.C.A. EMU 3 B. Les micrographies montrent

¹ La plage de Moustierlin, près de Concarneau, nous a fourni en abondance le matériel utilisé pour cette étude.

nombre de détails nouveaux concernant en particulier l'appareil flagellaire, le stigma et les chromoplastes, le corps de Golgi [14]. L'interprétation des structures observées suppose la confrontation et la comparaison des images électroniques données par *C. psammobia* avec celles précédemment obtenues

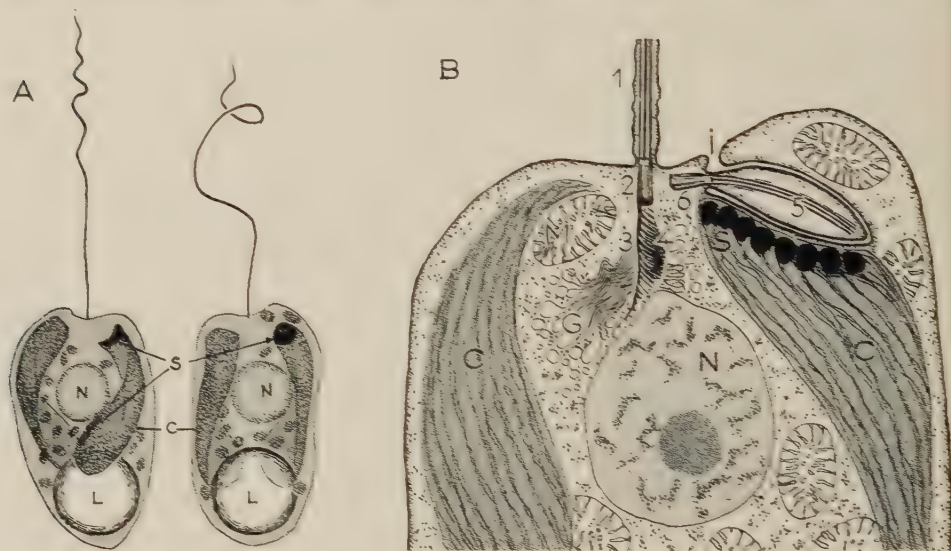


Fig. A. — *Chromulina psammobia*: aspect du Flagellé examiné à l'état vivant: N, noyau; C, chromoplastes; S, stigma apical et stigma accessoire; L, goutte de leucosine.

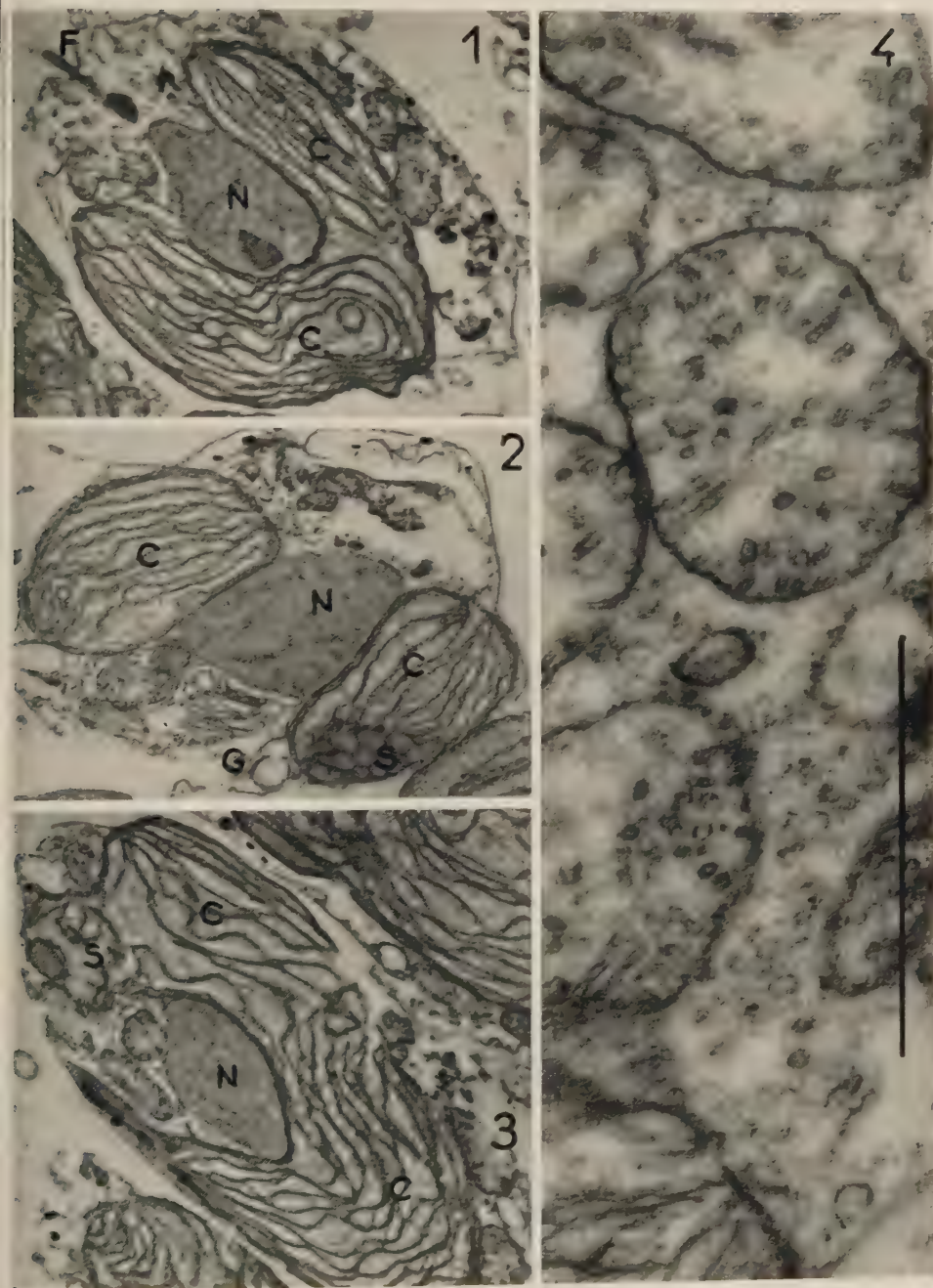
Fig. B. — Région antérieure de *Chromulina psammobia*: schéma combinant les indications apportées par diverses micrographies électroniques. Les lettres et les chiffres correspondent à ceux portés sur les planches, soit: N, noyau; C, chromoplastes; S, stigma; M, mitochondries; G, corps de Golgi; 1, invagination de la pellicule périphérique tapissant la chambre du flagelle interne; 2, flagelle externe; 3, son cinétosome; 4, rhizoplaste; 5, masse dense (centrosome?); 6, flagelle interne; 7, son cinétosome.

par divers auteurs et concernant soit des Phytoflagellés tels que *Euglena gracilis* [62, 63], les Chrysomonadiens *Poteriochromonas stipitata* [62, 63] et *Synura caroliniana* [42], un *Chlamydomonas*: *C. Reinhardi* [55], soit des Zooflagellés parasites tels que *Trichomonas muris* [1], *Joenia*, *Foaina*, *Trimitus*, *Spirotrichonympha*, *Trichonympha* [25], *Pyronympha* [26].

Le Flagellé chrysomonadien psammique examiné ici (Fig. A dans le

Fig. 1, 2, 3. — Quelques sections de *Chromulina*: micrographies à faible grossissement montrant les relations topographiques entre le noyau N, les chromoplastes C, le flagelle externe F, le stigma S (avec la section du flagelle interne visible sur Fig. 3), le corps de Golgi G.

Fig. 4. — Quelques mitochondries montrant les villosités internes d'aspect tubulaire et leur insertion sur la membrane interne.



texte) est de forme approximativement ovoïde ou piriforme; légèrement aplati transversalement, il mesure 13 à 15 μ de long sur 6 à 8 μ de large. La présence d'un seul flagelle visible, long de $\sim 20 \mu$, de deux chromatophores latéralement allongés et incurvés de part et d'autre du noyau, d'un stigma cupuliforme, et d'une goutte postérieure de leucosine, permettent de le classer dans le genre *Chromulina* Cienkowski (voir [36, 17, 22] etc.). Parmi les nombreuses espèces de ce genre (voir [35]) notre *Chromulina* se rapproche étroitement de *C. flavicans* Ehrb. (d'après [36]) et de *C. lunaris* N. Carter [3]; mais l'identification ne semble possible ni avec la première qui est dulçaquicole, ni avec la seconde, sensiblement plus petite; de ce fait elle a été décrite comme espèce nouvelle [12] sous le nom de *C. psammobia* qui rappelle ses caractères écologiques.

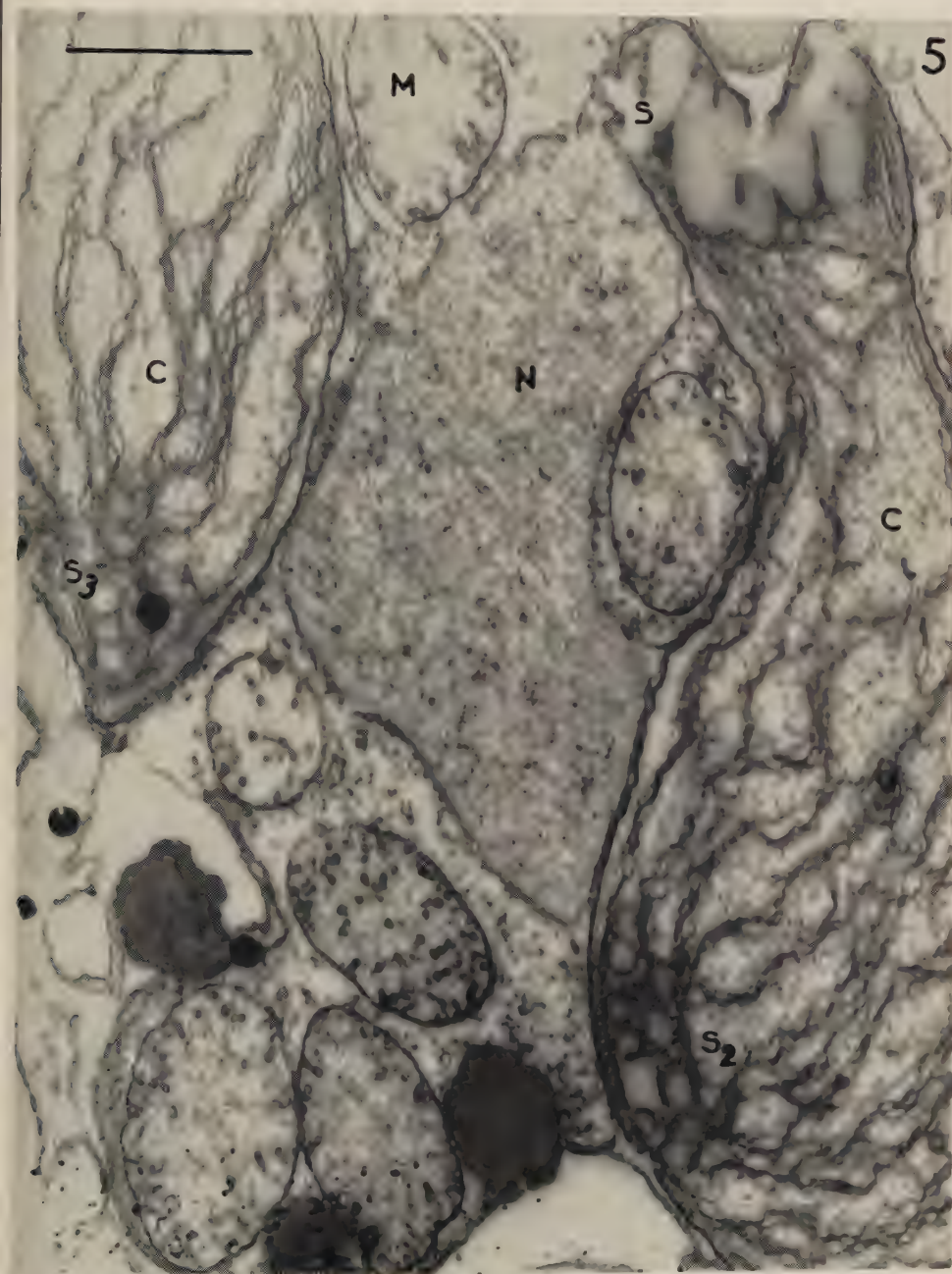
Cytoplasma et noyau

La masse cytoplasmique du Flagellé est enveloppée par une fine pellicule dont la section apparaît comme un trait sombre, régulier, continu, épais de moins de 100 Å, semblant parfois doublé extérieurement par une autre pellicule moins dense. Il est difficile de décider s'il n'existe en fait qu'une pellicule superficielle simple, ou bien si la condensation cytoplasmique superficielle est elle même recouverte par une pellicule cellulosique.

Le cytoplasma proprement dit comporte : (1) une substance fondamentale dont l'aspect finement et irrégulièrement granuleux est dû, sans aucun doute, à l'effet coagulant du réactif fixateur, et (2) divers éléments figurés tels que : (a) des granules mesurant 25 à 30 m μ de diamètre dont la nature est indéterminée; (b) des vésicules mesurant 60 à 100 m μ et dérivant peut-être du corps de Golgi; (c) quelques vacuoles très étroites et très allongées, sortes de fentes ou de doubles membranes qui pourraient appartenir à un « reticulum endoplasmique » ou ergastoplasme; (d) les mitochondries enfin, bien visibles en contraste de phase dans le Flagellé vivant, et montrant sur les micrographies électroniques (Fig. 1, 2 et 3) la structure interne bien connue (Fig. 4).

Les mitochondries de *Chromulina* mesurent 1 à 2 μ de long sur 0.6 à 0.7 μ de large; elles sont limitées par une membrane assez dense, épaisse de près de 200 Å et probablement complexe; c'est à partir de la face interne

Fig. 5.—Section passant par le noyau N, les deux chromoplastes C, le stigma S avec sa concavité externe et la coupe transversale du flagelle interne. Noter la structure lamellaire des chromoplastes et la présence, chez celui de droite, d'un stigma secondaire postérieur S_2 et celle, sur celui de gauche, d'une différenciation vésiculeuse S_3 . Le cytoplasma granuleux renferme, autour et au dessous du noyau, des mitochondries et quelques masses sombres de nature lipidique.



de cette membrane que les digitations flexueuses, larges de 350 à 400 Å s'enfoncent dans la matrix amorphe qui constitue la masse de l'organite (Fig. 4).

Le cytoplasme avec ses inclusions et le chondriome occupe tout l'espace irrégulièrement compris entre les organites qui, tels le noyau, les chromoplastes, le corps de Golgi, les dérivés flagellaires, constituent la plus grande partie de la masse cellulaire.

Le noyau est un corps ovoïde, limité par une double membrane (Fig. 5, 8, 9, 10, 11). Les micrographies électroniques montrent dans le précipité très ténu correspondant au suc nucléaire des amas finement granuleux, étirés en travées irrégulières, souvent noduleuses, correspondant semble-t-il aux granules Feulgen positifs visibles au microscope optique; le nucléole Feulgen négatif visible dans les mêmes conditions est représenté par une masse beaucoup plus volumineuse, plus dense, de contour irrégulier (Fig. 1).

Chromoplastes et stigma

Les deux grands chromoplastes longitudinaux (Fig. 1, 2, 3) sont enveloppés chacun par une membrane continues, dense, épaisse de 100 Å environ (Fig. 5, 7, 8). La masse interne est constituée par un ensemble de doubles lamelles plus ou moins flexueuses, convergeant vers chacune des extrémités antérieure et postérieure d'un chromoplaste. L'espace, d'épaisseur variable, compris entre les cloisons bilamellaires est occupé par un fin granulum irrégulier (Fig. 5, 8).

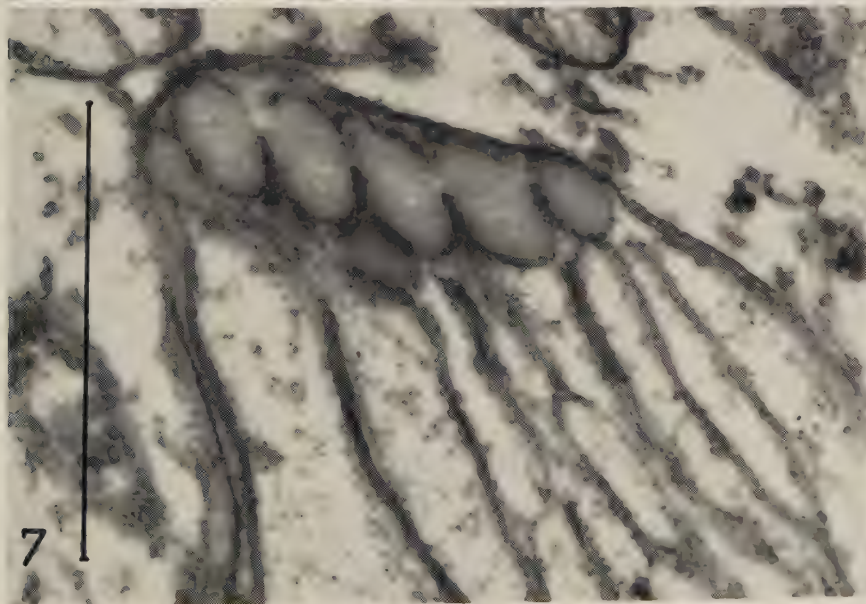
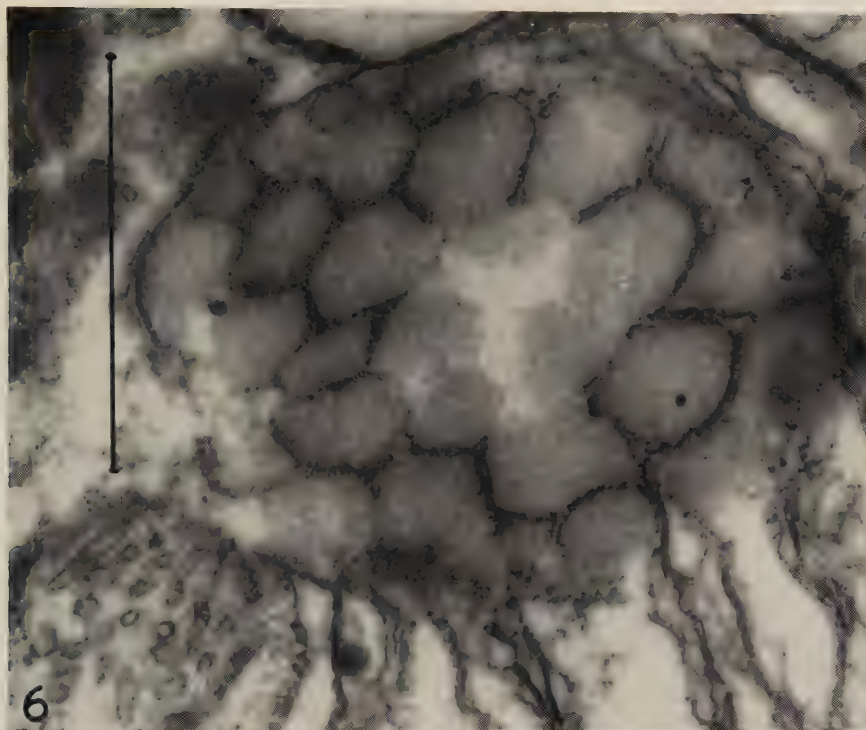
Aucun détail morphologique ne permet de caractériser valablement, chez *Chromulina psammobia*, une face ventrale et une face dorsale; de ce fait les deux chromoplastes ne peuvent être distingués comme droit ou comme gauche, et l'on retiendra seulement que l'un d'eux porte le stigma à son pôle antérieur, et, éventuellement, un stigma accessoire près de son pôle postérieur.

Le stigma antérieur apparaît au microscope optique comme une petite cupule réfringente fortement colorée en rouge par un pigment caroténoïde donnant avec la solution iodo-iodurée une teinte bleu violacé intense (Fig. A dans le texte).

Les micrographies électroniques montrent que cette cupule est incluse

Fig. 6.—Section tangente à la face profonde de la cupule stigmatique; les éléments vésiculeux coupés transversalement montrent leurs parois juxtaposées et leur contenu dense.

Fig. 7.—Section transversale du stigma montrant la juxtaposition des éléments vésiculeux ovoïdes situés au dessous de la membrane propre du chromoplaste.



dans le chloroplaste et recouverte par sa membrane; elle est constituée par la juxtaposition d'éléments vésiculeux ovoïdes, dont les deux diamètres mesurent respectivement 0.3 et 0.2 μ environ (Fig. 2, 5, 6, 7, 13, 14); ces éléments sont constitués par une substance homogène plus dense que celle du plaste; ils sont limités par un contour sombre qui paraît en continuité avec les lamelles chromoplastiques; mais ce contour, interrompu de place en place, semble laisser ainsi des communications intervésiculaires.

Bien que la bipartition des *Chromulina* n'ait pas été examinée, certains aspects suggèrent une néoformation du stigma. Il s'agit de chromoplastes montrant (Fig. 8) à l'un de leurs pôles une zone sombre au niveau de laquelle les cloisons bilamellaires contournées et pelotonnées, conservent un écartement régulier, les espaces qu'elles délimitent étant remplis par une substance dense et finement granuleuse. D'autres images montrent comme une constriction du système lamellaire qui tend à isoler la zone dense sous la membrane du chromoplaste, puis un cloisonnement de cette zone qui paraît annoncer la formation des éléments vésiculeux, encore incomplètement isolés les uns des autres.

La coaptation de la cupule stigmatique antérieure avec le flagelle interne est examinée plus loin. Notons ici que le stigma postérieur, lorsqu'il existe, est de forme moins définie, et que l'on peut observer (Fig. 5) quelques aspects d'une différenciation stigmatique fruste sur l'autre chromoplaste.

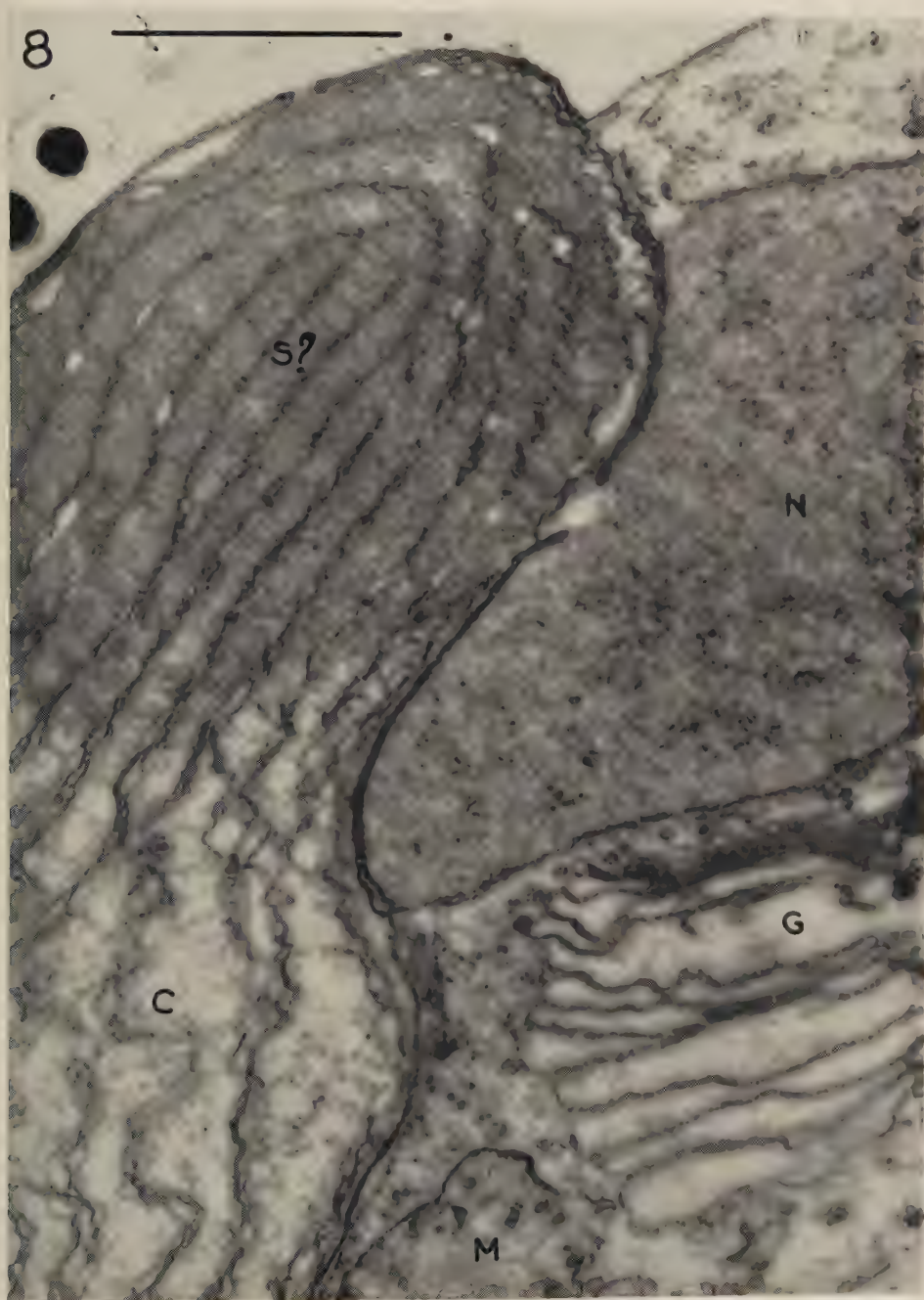
Flagelle externe et complexe cinéto-rhizoplastique

L'observation de *Chromulina psammobia* au microscope optique montre la présence d'un seul flagelle, ce qui répond à l'un des caractères du genre. Par contre, le microscope électronique démontre qu'il existe en fait deux organites de nature ciliaire, soit un grand flagelle visible qu'on dénommera *externe* et un très court flagelle invisible parce qu'intracytoplasmique, que l'on dénommera *interne*, et dont il sera question plus loin.

Le flagelle externe (Fig. A dans le texte) se dresse à l'extrémité antérieure du corps et sa longueur atteint environ 20 μ ; il montre au microscope électronique la structure bien connue des organites vibratiles, soit le faisceau cylindrique de neuf fibrilles périphériques entourant une double fibrille centrale, et la membrane enveloppante qui apparaît comme une extension de la pellicule cellulaire¹.

¹ En ce qui concerne la structure des flagelles, voir Pitelka et Schooley [49].

Fig. 8.—Section passant par le noyau *N*, le corps de Golgi *G*, et l'un des chromoplastes *C* dont le système lamellaire présente une curieuse disposition régulière et concentrique, enrobant un contenu dense et homogène et représentant peut être l'ébauche d'un stigma *S*?



La partie intracytoplasmique du flagelle externe comprend le corpuscule basal et le dispositif radiculaire. Le corpuscule basal, ou cinétosome, de forme tubulaire, mesure près de 0.5μ de haut et environ $130 \text{ m}\mu$ de large; les neuf fibrilles périphériques du flagelle se prolongent dans l'épaisseur de sa paroi, tandis que la double fibrille centrale s'arrête un peu au dessus sur un corpuscule dense et plat (Fig. 10 et 13).

Le tube cinétosomien est prolongé sur l'un de ses côtés, et en direction postérieure, par une pièce radiculaire ou rhizoplaste. Ce rhizoplaste est une mince lamelle incurvée en forme de gouttière, portant des épaisissements obliques régulièrement espacés; la période mesurée suivant les génératrices de cet organe est d'environ $425 \text{ \AA}$ (Fig. 9, 10, 13).

La gouttière rhizoplastique descend sur une hauteur de 1μ environ, jusqu'au noyau, dont elle coiffe latéralement le pôle antérieur proéminent en forme de cône; aucun contact direct n'est visible entre la membrane nucléaire et la structure radiculaire qui s'incurve à son niveau, puis disparaît dans le cytoplasme environnant.

La concavité de la gouttière rhizoplastique est partiellement occupée par une masse allongée, dense, au contour estompé; il est possible que ce soit le centrosome (Fig. 9, 10, 13).

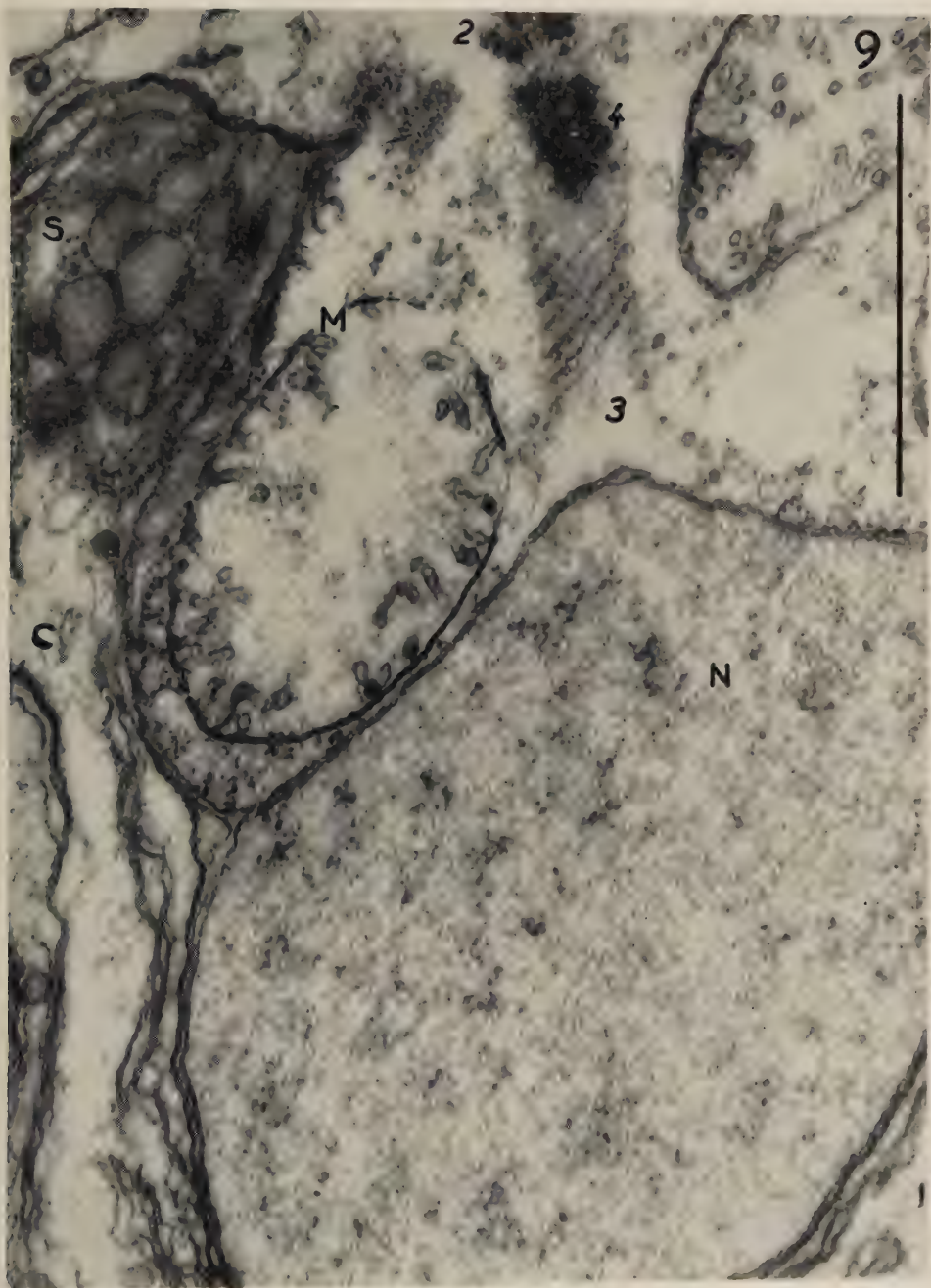
Le corps de Golgi est situé dans le cytoplasme entre le chromoplaste astigmatique, le noyau et la face convexe du rhizoplaste. Il est composé d'une pile d'éléments bilamellaires plus ou moins incurvés, constituant autant de saccules aplatis dont les bords se dilatent en larges ampoules; celles-ci semblent se fragmenter elles-mêmes en petites vésicules, bientôt répandues dans le cytoplasme tout à l'entour (Fig. 8, 10, 11).

Il n'existe aucune apparence de connexion directe entre ce corps de Golgi et le dispositif radiculaire, mais le groupement des divers organites précités est si étroitement localisé entre la base du flagelle externe et la surface nucléaire, que l'on peut dénommer leur ensemble « complexe cinéto-rhizoplastique » (Fig. B dans le texte; Fig. 10).

Flagelle interne et complexe cinéto-stigmatique

Quelques sections favorables montrent auprès de la base du flagelle externe, vers la moitié de la hauteur du cinétosome, et perpendiculaire à celui-ci, un

Fig. 9.—Section passant par la base du flagelle externe avec une partie de son cinétosome 2, le rhizoplaste 3 (constitué par une lamelle obliquement striée qui s'élargit au voisinage du noyau N), et la masse dense 4 correspondant peut-être au centrosome. Noter la structure interne du noyau marquée par des condensations irrégulières (correspondant à des granules Feulgen-positifs) et la double membrane qui l'enveloppe. A gauche, chromoplaste C à structure lamellaire et section oblique du stigma S.



second cinétosome tubulaire; disposé à peu de distance au dessous de la membrane externe et parallèlement à celle-ci, il est orienté vers le chromoplaste stigmatique (Fig. B et 12, 13).

Le bord postérieur de ce second tube cinétosomien est légèrement épaissi et ne montre aucun prolongement radiculaire; au niveau de son extrémité antérieure, la membrane cellulaire externe s'enfonce (Fig. 12 et 13) à travers un étroit pertuis, prend contact par l'un de ses bords avec le col cinétosomien, et son invagination assure tout à la fois la formation de la membrane ciliaire et le revêtement de la chambre dans laquelle se trouve enfermé le flagelle interne. Cette chambre s'insère exactement dans la dépression de la cupule stigmatique recouverte par la membrane propre du chromoplaste. Elle est occupée par la masse du flagelle interne.

Le flagelle interne paraît être un organite hautement spécialisé; sa longueur, très courte, ne semble guère dépasser $1\ \mu$; il contient un faisceau fibrillaire dont les neuf fibrilles périphériques apparaissent dédoublées dans le sens radial (Fig. B et 14, 16, 17); ce faisceau est inclus dans une matrix amorphe. Du fait de l'hypertrophie de cette matrix le flagelle interne affecte la forme d'un ovoïde allongé, dont l'épaisseur atteint près de $0.5\ \mu$ dans la région centrale.

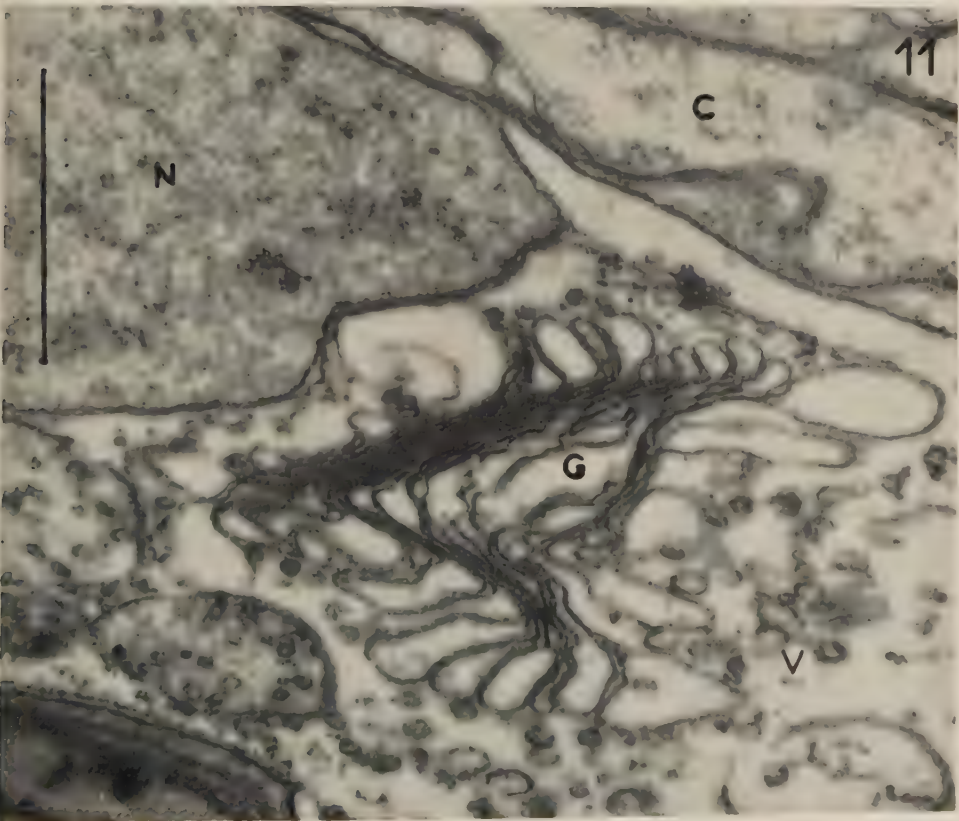
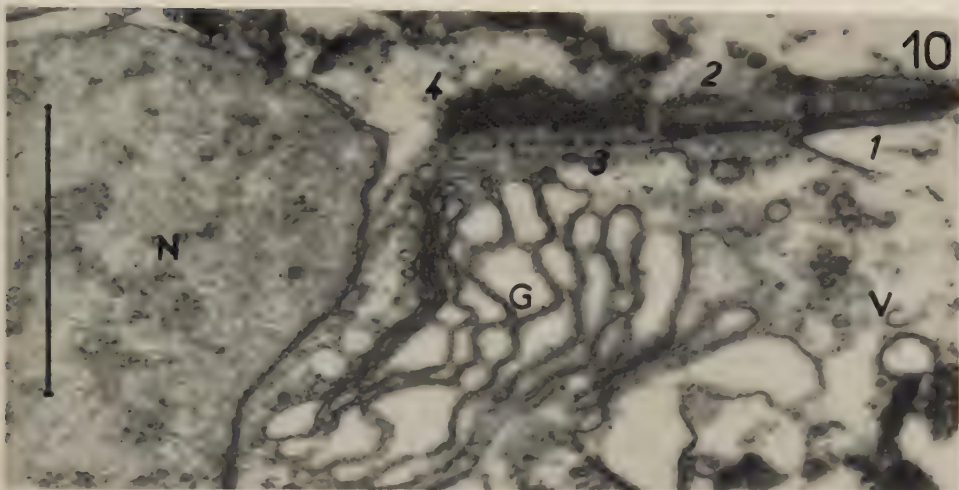
La coaptation flagelle interne-stigma semble comporter seulement des relations de contiguité, et l'on notera l'interposition constante de trois membranes: celle du chromoplaste, tapissant la cupule stigmatique; celle de la chambre flagellaire intracytoplasmique; celle du flagelle, enfin, limitant la matrix qui entoure le faisceau fibrillaire. Cet ensemble flagello-stigmatique suggère évidemment l'idée d'un organite photo-sensible.

Comparaisons et discussion

L'étude au microscope électronique du Flagellé chrysomonadien *Chromulina psammobia* apporte peu d'informations nouvelles relatives aux constituants ordinaires de toute cellule. On notera, par exemple, que la structure intime des mitochondries est caractérisée chez cette espèce, comme chez la plupart des Protistes [53, 54, 56], par la présence de villosités digiti-

Fig. 10.—Section intéressant dans son ensemble le complexe cineto-rhizoplastique. 1, base du flagelle externe; 2, son cinétosome tubulaire prolongé par la lamelle rhizoplastique 3, (section sagittale) dirigée vers le noyau N, et portant sur sa face concave la masse dense 4. (centrosome?). G, corps de Golgi et vacuoles V résultant de la fragmentation marginale des saccules aplatis (voir fig. 11).

Fig. 11.—Corps de Golgi, G, montrant l'empilement bilamellaire des saccules aplatis, la dilation ampullaire de leur zone marginale et la fragmentation de celle-ci en petites vacuoles V.



formes et non point de crêtes, fait déjà reconnu chez un autre Phytoflagellé du genre *Synura* [42]. De même la structure feuilletée des chromoplastes [37, 38, 39, 40, 30, 31, 60, 59, 6] est bien connue chez divers Chrysomonadiens et Eugléniens (Wolken et Palade [62, 63], Sager et Palade [55], Wolken [64]) mais on n'observe pas chez *C. psammobia* de zones lamellaires condensées pouvant correspondre à des « grana » ou bien à un pyrénioïde. Enfin l'organisation des deux flagelles avec leur faisceau fibrillaire reste, malgré la curieuse différenciation du flagelle interne, conforme au type universel à 9 + 2 fibrilles.

Par contre, les micrographies électroniques apportent d'intéressantes données concernant les deux groupements d'organites différenciés décrits dans les pages précédentes sous les noms de complexes cinéto-rhizoplastique et cinéto-stigmatique.

On reconnaît aisément dans le complexe cinéto-rhizoplastique lié au flagelle externe nombre d'aspects structuraux visibles au microscope optique chez divers Flagellés soumis à des colorations adéquates; il apparaît cependant qu'à l'échelle de la microscopie électronique l'interprétation des structures observées peut réserver quelques incertitudes.

Le terme « rhizoplaste » désigne un filament connectif étendu chez nombre de Flagellés, entre les corps basaux des organites vibratiles (blépharoplastes ou cinétosomes) et le noyau. Le ruban obliquement strié qui, chez *Chromulina*, prolonge sur un côté la paroi du cinétosome, peut-être considéré d'après cette connexion, comme une racine ciliaire; en fait c'est bien par ce terme que Manton [42] désigne chez *Synura*, une structure probablement identique à celle de *Chromulina* si l'on considère à la fois sa position et son « diagonal cross marking ».

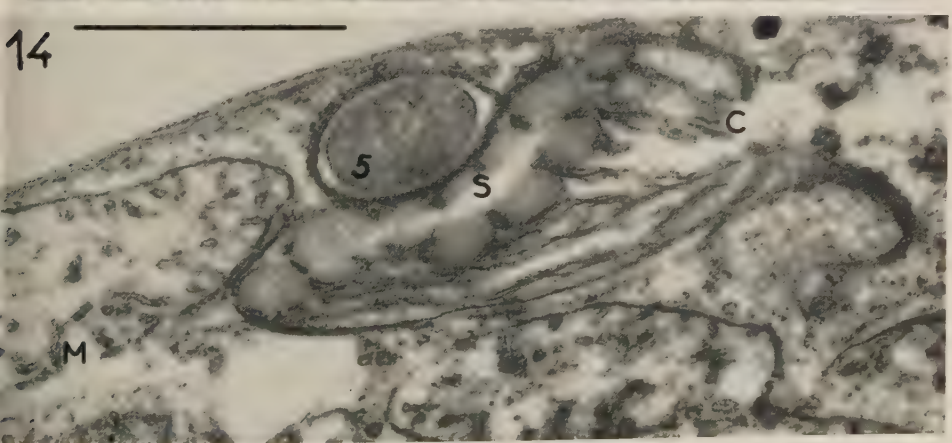
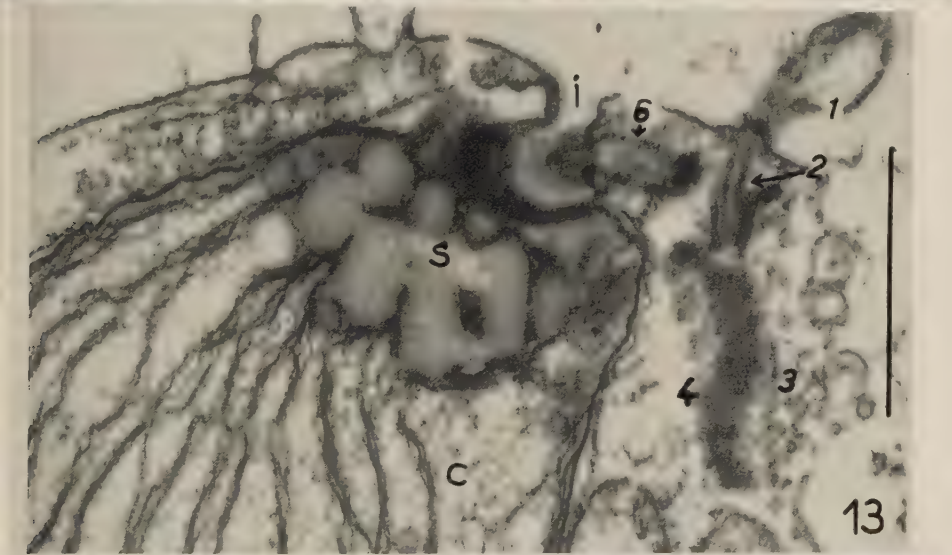
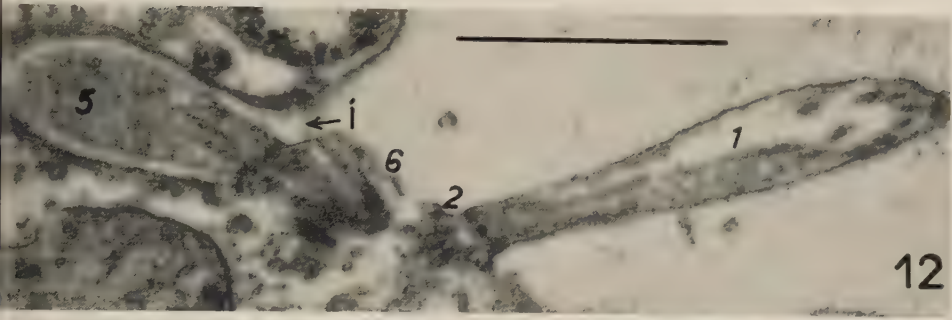
Notons d'autre part que le « ruban axial » décrit par Grassé [26] chez un Zooflagellé parasite, *Pyronympha vertens*, pourrait être homologue des formations radiculaires¹ observées chez les deux Chrysomonadiens précé-

¹ La microscopie électronique des Ciliés montre que les racines ciliaires formées à partir des cinétosomes peuvent réaliser des structures remarquablement et diversement complexes [12, 13, 51].

Fig. 12.—Section intéressant la base des deux flagelles. 1, flagelle externe en coupe oblique montrant les fibrilles du faisceau axial; 2, une partie de son cinétosome; I, invagination de la paroi du corps formant la chambre du flagelle interne 5 dont une partie du cinétosome 6 est visible.

Fig. 13.—Section montrant les deux complexes cinéto-rhizoplastique: 1, 2, 3 et 4, et cinéto-stigmatique: 6, I et S. — 1, flagelle externe; 2, son cinétosome; 3, lamelle rhizoplastique striée obliquement; 4, masse dense (centrosome?). I, invagination formant la chambre du flagelle interne; 6, le cinétosome de celui-ci; S, stigma, C, chromoplaste.

Fig. 14.—Section transversale du flagelle interne 5 à l'intérieur de la chambre flagellaire (mince espace clair) limitée par l'invagination de la membrane externe. S, stigma; C, chromoplaste.



dents. Dans les trois cas, les rapports du « rhizoplaste » avec le noyau semblent identiques; le ruban ou les fibres radiculaires se dirigent directement vers le pôle antérieur du noyau puis s'infléchissent au voisinage immédiat de sa surface et la contournent de près sur une certaine longueur. Aucune connexion directe avec la membrane nucléaire n'est visible, mais, bien qu'une liaison mécanique imaginée par Manton comme un « ancrage des bases ciliaires sur le noyau », ne soit pas démontrée, il est remarquable que chez *Pyrsonympha*, comme chez *Synura* et chez *Chromulina* le pôle antérieur du noyau se soulève en forme de cône à l'approche du rhizoplaste.

La signification du corps dense allongé chez *Chromulina* dans la gouttière rhizoplastique reste problématique; l'hypothèse de sa nature centrosomienne est suggérée par des éléments de comparaison encore insuffisants, et ne pourrait être valablement discutée que devant quelques images de division qui font actuellement défaut. Chez *Pyrsonympha*, Grassé [26] décrit le centrosome comme un corps ellipsoïde homogène, assez dense, sur lequel s'insèrent les corps basaux des flagelles récurrents, le ruban axial et le paraxostyle; les micrographies montrent le paraxostyle allongé sur le côté du ruban axial, et l'aspect de ce dérivé centrosomien très osmiophile pourrait évoquer celui du corps rhizoplastique dense de *Chromulina*.

Le corps de Golgi observé chez *Chromulina* sur un côté du rhizoplaste et du noyau est exactement comparable au « lamellated organ » décrit chez *Synura* par Manton [42], comme chez *Trichomonas muris* par Anderson [1]; il en existe plusieurs chez les Euglènes où Wolken et Palade les avaient préalablement décrits [63] comme représentant le « réticulum endoplasmique ».

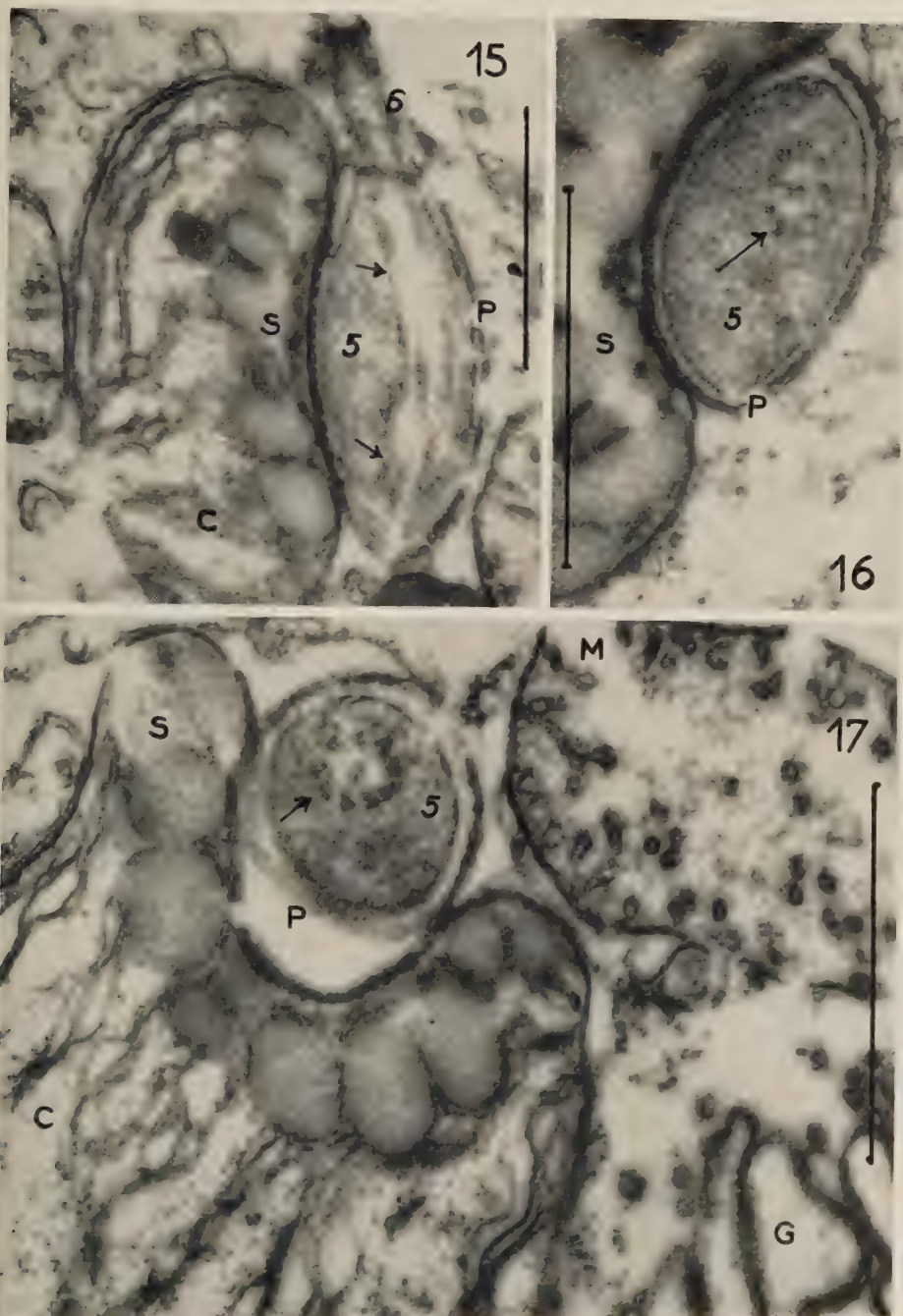
Il est inutile de rappeler la longue histoire de l'appareil de Golgi, ni les interprétations diverses dont il a été l'objet, car la microscopie électronique a renouvelé les données du problème et la documentation nécessaire sera trouvée dans les publications de Bernhard, Gautier et Rouiller [2]; Haguénau et Bernhard [27, 28]; Palade [48]; Sjöstrand [59] etc.

Il faut savoir distinguer cependant entre certaines cytomembranes de Sjöstrand, le réticulum endoplasmique de Porter avec des grains ergasto-

Fig. 15. Section longitudinale marquant du flagelle interne 5 et de son cinétosome 6; les deux flèches le faisceau fibrillaire. La chambre flagellaire est limitée par l'invagination de la pellicule externe P et repose sur la membrane du chromoplaste C au dessus du stigma S.

Fig. 16.—Section oblique du flagelle interne.

Fig. 17.—Section transversale du flagelle interne 5. La flèche montre le faisceau fibrillaire. La pellicule périphérique P est décollée (accidentellement) de la membrane propre du chromoplaste, bien visible au dessus des vésicules stigmatiques.



plasmiques de Palade, et les saccules lisses et plats constituant selon Dalton [7], Dalton et Felix [8, 9] l'appareil de Golgi, avant de reconnaître que les organites feuilletés de *Euglena*, *Synura*, *Trichomonas* et *Chromulina*, de même que ceux décrits par Grassé [26] chez quelques Zooflagellés, montrent tous les caractères structuraux et tous les signes d'activité élaboratrice des systèmes golgiens.

Chez les Euglènes, de tels corps sont dispersés dans le cytoplasme, entre les chloroplastes, et correspondent probablement aux dictyosomes mis en évidence par Hollande [32]; chez les autres Flagellés chrysomonadiens le corps de Golgi, unique; occupe une position définie près du noyau et du rhizoplaste. Aucune connexion ne semble ici le relier directement soit au rhizoplaste, soit au supposé centrosome ou au cinétosome; l'identification de cet organite golgien avec un corps parabasal, proposée par Grassé [26] dans le cas de quelques Zooflagellés parasites, ne se justifierait donc, chez les Flagellés chrysomonadiens, que par la situation de cet organite dans l'ensemble structural précédemment dénommé complexe cinéto-rhizoplastique.

On a vu comment, chez *Chromulina*, le stigma est situé dans un chromoplaste, juste au dessous de la membrane propre de celui-ci; l'examen au microscope électronique d'un *Chlamydomonas* [55] et du spermatozoïde de *Fucus* [43] avait apporté déjà deux exemples de ce fait, vérifiant ainsi les résultats d'observations faites au microscope optique. Dès 1914, en effet, Rothert [52] affirmait que la tache oculiforme des Phytoflagellés est de nature chromoplastidienne. Réfutant la notion proposée par Grassé [18, 19, 20] puis abandonnée par Grassé et Hollande [21], que le stigma est un corps parabasal, Mangenot [41], puis Chadefaud [4, 5], Hollande [32, 33, 34] etc., précisaient que chez les Chlorophycées et les Xanthophycées, le stigma est une partie différenciée d'un chromoplaste, tandis que chez les Euglènes il résulte de la différenciation totale de l'un de ces organites.

On doit encore à la microscopie électronique de connaître la complexité structurale du stigma. Chez *Euglena gracilis* [63, 64], *Chlamydomonas reinhardi* [55], le spermatozoïde de *Fucus*, comme chez notre *Chromulina psammobia* et chez un *Chlamydomonas* symbiote du *Strombidium oculatum* ([10, 11] et observations non publiées), le stigma est constitué par la juxtaposition d'éléments ou de « chambres » sphéroïdes, ovoïdes, ou cylindroïdes dont les diamètres sont approximativement compris entre 100 et 400 m μ . Ces éléments contiennent le pigment caroténoïde, qui est aisément extrait par l'alcool après fixation au peroxyde d'osmium à pH 8,2 et déshydratation lente, mais très peu soluble après fixation neutre et déshydratation rapide (Manton et Clarke); il est possible que ce pigment soit responsable de l'aspect

dense montré, sur les micrographies, par le contenu des « chambres » stigmatiques.

Un fait remarquable est apporté par la coaptation flagello-stigmatique. Celle-ci était précédemment soupçonnée chez les Euglènes; chez *E. gracilis* Wolken et Palade [63] ont précisé la présence d'un corps dense, homogène, disposé face au stigma, et relié à l'un des flagelles, de sorte que l'on peut l'homologuer au « corps paraflagellaire », considéré comme un photo-récepteur. Chez le spermatozoïde de *Fucus* la coaptation est beaucoup plus étroite d'après Manton et Clarke [43]; tandis que le flagelle antérieur reste entièrement libre, le flagelle récurrent se recourbe, et prend contact avec la surface du corps au niveau de la tache oculiforme; suivant toute la largeur de celle-ci il adhère à la membrane cellulaire sur laquelle il étale sa propre membrane, tandis que, au delà, il redevient libre et normalement cylindrique.

Comme le soulignent Manton et Clarke, la signification fonctionnelle de cette curieuse adhérence d'un flagelle à la membrane du corps au dessus du stigma est inconnue; mais la dilatation corrélative de la membrane flagellaire n'est pas un artefact, car l'espace intérieur est entièrement rempli par une matrix homogène dans laquelle le faisceau fibrillaire axial occupe une position excentrée vers le dehors.

Le cas de *Chromulina psammobia* est exactement comparable à celui du spermatozoïde de *Fucus*, à ceci près que le complexe cinéto-stigmatique est beaucoup plus étroitement différencié; rappelons en effet que le flagelle associé à la tache oculiforme se réduit chez *Chromulina* à un court segment entièrement inclus dans une étroite gaine intracytoplasmique, cependant que la coaptation s'effectue, comme chez le *Fucus*, à travers l'épaisseur de trois membranes, entre la partie dilatée du flagelle et la surface incurvée du stigma qui la moule étroitement.

Le stigma des Phytoflagellés est généralement considéré avec Franzé [15, 16], Rothert [52], Mast [44, 45, 46], comme une « tache oculiforme », c'est-à-dire un organite sensoriel. Pour Wolken [64, 65], le complexe stigma-flagelle est un appareil photorécepteur dont la structure évoque celle décrite par Sjöstrand [59] pour le segment externe des cellules visuelles des Vertébrés, et les interprétations générales proposées par Wilmer [61].

On connaît chez *Pyrsonympha* [26] la coaptation des flagelles récurrents avec la surface du corps, mais celle-ci ne semble pas liée à une différenciation fonctionnelle particulière.

La présence chez une espèce du genre *Chromulina* d'un second flagelle, masqué par sa situation intracellulaire, s'accorde avec différents aspects réalisés par la différenciation et l'évolution de Phytoflagellés, et avec les

conclusions d'un important travail que P. Bourrelly consacre à ces problèmes sous le titre « Recherches sur les Chrysophycées; Morphologie, Phylogenèse, Systématique ».¹

Etant donné le haut degré de spécialisation atteint par le flagelle interne, il eut été intéressant de savoir si, comme dans la plupart des cas comportant une différenciation flagellaire, c'est le flagelle principal qui porte des mastigonèmes. Les colorations nécessaires non plus que les frottis ombrés pour la microscopie électronique n'ont pas été réalisés; mais l'examen des sections ultrafines apporte différentes indications relatives à la présence, sur le flagelle externe, de mastigonèmes latéraux.

RÉSUMÉ

Chromulina psammobia est une Chrysomonadale pourvue de deux flagelles, l'un est externe et seul visible à l'état vivant; l'autre est interne et n'a pu être décelé que par le microscope électronique.

Une lamelle rhizoplastique obliquement striée prolonge jusqu'au noyau le cinétosome du flagelle externe; elle est associée à un corps dense qui pourrait être un centrosome, et à un appareil de Golgi.

Le flagelle interne est inclus dans une chambre constituée par une invagination de la pellicule ectoplasmique; très court et dilaté, il est en étroite coaptation avec le stigma.

Le stigma est inclus dans le chromoplaste dont il représente une partie différenciée; il est constitué par la juxtaposition de corps vésiculeux contenant le pigment caroténoïde.

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THE FORMATION OF GLUTATHIONE AND THE DEVELOPMENT OF SOME ENZYMES FOR CARBOHYDRATE METABOLISM DURING THE DEVELOPMENT OF CHICKEN EMBRYO¹

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THE importance of sulfhydryl groups in cell division and in cellular growth has been recognized since the pioneer investigations of Rapkine [23], who discovered that the concentration of -SH groups in sea urchin eggs diminished soon after fertilization, reaching its lowest value 30 minutes later, and increased suddenly afterwards to reach a maximum 45 minutes after fertilization. The recent findings of Mazia [13] and of Stern [29] have confirmed essentially Rapkine's observations. The increase of -SH groups during the process of cellular growth has also been found in plants, and in protozoa (see review by Barron [1]). The chicken embryo is well suited for these studies because of its size, and the thorough documentation which provides a definite correlation between cell structure and age of development. A number of investigators have measured the amount of -SH groups of chicken embryo during development with contradictory results. Castagna and Talenti [4] reported that glutathione was not present in mature hen eggs, but that it was formed during incubation, very slowly during the first 10-12 days, rapidly afterwards. Talenti [31] later reported that -SH groups could be detected histochemically in the various portions of the hen's egg, both fertile and unfertile, a histochemical detection applied more thoroughly by Buño [2]. According to Muratta [17] the glutathione content of the yolk of fertilized egg is slightly higher than that of the unfertilized egg, the content decreasing as incubation proceeds; in the embryo, he claimed, glutathione increased. An indication of glutathione synthesis was obtained by Muratta

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and Sagara [27] after the injection of glutamic acid. Yaoi [34] found that the glutathione content of developing chicken embryos increased gradually, reaching its maximum in 12–14 days, after which it decreased. The main objection to all these investigations is that the methods used for the determination of glutathione (mostly by iodine titration) were not specific for -SH groups or glutathione, and were not sufficiently sensitive.

We present in this paper the results of our investigations on the glutathione content of chicken embryo during development, together with the measurement of the activity of enzymes concerned with the reduction of oxidized glutathione and cystine, and of some enzymes for carbohydrate metabolism.

METHODS

Fresh hen's eggs were incubated under appropriate conditions of aeration, temperature, and humidity, the state of development being determined according to the tables of Lillie [11]. For the determination of -SH groups, the embryos were separated from the yolk and the membranes were weighed rapidly and dropped into a glass homogenizer containing 0.376 *M* trichloroacetic acid (3 ml for 100–200 mg fresh weight). After homogenization, an aliquot was taken for *N* determination, and the remaining suspension was centrifuged in a refrigerated centrifuge at 0°C. Glutathione was determined in the supernatant fluid according to Grunert and Phillips [6]. The samples were kept at 4°C for 20 minutes for color development, and the color intensity was measured at 520 m μ in a Beckman spectrophotometer with temperature-controlled cells at 4°C. Under these conditions the method is very reliable, although not specific for glutathione. The glutathione content of yolk, egg white, and whole egg was measured after trichloroacetic acid precipitation. When turbid solutions were obtained, the samples were centrifuged again in the Spinco preparative ultracentrifuge for 30 minutes at 30,000 rpm; when the solutions became turbid after the addition of NaCl they were filtered prior to spectrophotometric readings. In embryos, glutathione was determined from 65 hours to 278 hours after incubation; during the first hours of incubation, it was measured in samples made by pooling several embryos of the same stage of development.

Enzyme activity was measured in cell-free extracts prepared by homogenization of the embryos in 0.154 *M* KCl and centrifugation at 0° for 40 minutes at 30,000 rpm. The experiments on metabolism were performed with the usual Warburg manometric technique. Nitrogen was determined by the Kjeldahl microdistillation method of Ma and Zuazaga [12]; ribose, by Mejbaum's method [14]; heptulose, Horecker *et al.* [9]; fructose, Roe [25], GSH, GSSG, barium hexose diphosphate, and ribose-5-phosphate were obtained from Schwarz and Co., New York; DPN⁺,¹ TPN⁺, from Pabst Laboratories, Milwaukee, Wisconsin; the reduced compounds were prepared according to Hogeboom and Barry [7].

¹ The following abbreviations are used: GSH, reduced glutathione; GSSG, oxidized glutathione; DPN⁺, diphosphopyridine nucleotide; DPNH, reduced DPN⁺; TPN⁺, triphosphopyridine nucleotide; TPNH, reduced TPN⁺; R-5-P, ribose-5-phosphate; Tris, tris (hydroxymethyl) aminomethane.

RESULTS

The glutathione content of hen's eggs.—Hen's eggs before incubation contained glutathione, all of which was found in the yolk. The average glutathione content was about 3.05 micrograms per gram of egg, or 11.6 micrograms per g of yolk. By the end of 15 days' incubation it had risen to 45 micrograms per gram of egg, a net increase of 14 times the initial value—evidence of large synthesis (Table I). This increase was not clearly established until six days after incubation.

Since the nitroprusside reaction gives only a measure of $-SH$ compounds and is not specific for glutathione, it was necessary to establish the nature of the sulfhydryl compound present both in the egg and in the embryo. The yolk and the albumen were diluted with one volume of glass-distilled water free of traces of metals. Embryos and membranes were also diluted in the same manner. After homogenization, the samples were centrifuged in the Spinco ultracentrifuge for 30 minutes at 40,000 rpm. The non-fatty portion of the supernatant fluid was ultrafiltered according to Muntz and Barron [16] to obtain a protein-free filtrate. These samples, as well as standard solutions of reduced and oxidized glutathione, were treated with 0.1 *M* neutral KCN (0.9 ml of sample + 0.1 ml KCN), and 15 λ were put into previously washed Schleicher and Schull No. 507 filter paper. The solvent employed was *N*-propanol, formic acid, diethylglycol, water (70-10-3-20). The chromatograms were developed at 30°C, 45 per cent humidity, for 24 hours. At the end of the run the paper was dried at room temperature. The ninhydrin reagent (0.1 per cent ninhydrin in 90 per cent isopropanol containing 4 mg

TABLE I. *The total glutathione content of chicken eggs.*

Incubation h	Egg weight g	GSH μ g
0	49.46	172.0
0	50.33	134.4
0	60.26	193.5
0	53.08	150.0
94	50.86	132.35
162	48.23	233.0
211	51.08	452.5
240	51.58	660.0
358	53.16	2,438.5

per cent stannous chloride) was sprayed and dried in warm air. The paper was kept for 60–75 minutes at 30°C, and was kept in a dark place overnight. Under these conditions the R_f values of GSSG and GSH were 0.38 and 0.64 respectively. As can be seen in Fig. 1, the spots found in the ultrafiltrates of whole egg, of the whole egg after eight days' incubation, and of the chicken embryo coincided with those of reduced glutathione.

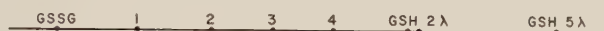


Fig. 1.—Paper chromatography of the ultrafiltrate of chicken embryo and of egg extracts (ninhydrin reaction). 1, Whole egg before incubation; 2, same; 3, embryo, 8 days old; 4, whole egg, 8 days after incubation.



Further demonstration that the $-SH$ groups of the protein-free filtrates of chicken embryo are glutathione, was obtained with the use of the glyoxalase method of Woodward [33], improved by Racker [21], so that it can measure amounts as small as 3 micrograms. The glyoxalase method is specific for glutathione. Three separate determinations of the $-SH$ groups of chicken embryo filtrates (sulfosalicylic acid precipitation) were made, with the two methods: nitroprusside and glyoxalase. The embryos were 5, 6, and 7 days old. The values obtained (microgram glutathione in 0.5 ml filtrate) were respectively: 15.50, 20.5, and 18.68 micrograms by the nitroprusside method; 16.9, 20.5, and 18.57 micrograms by the glyoxalase method. These results added to those of paper chromatography are proof that the $-SH$ groups of the protein-free filtrates of chicken embryo are almost exclusively glutathione.

Glutathione content in the developing embryo.—For determination of glutathione in the chicken embryo, the embryo was carefully removed from its surroundings. The embryos were obtained at different periods of growth — from 68–72 hours to 279 hours after incubation. The amount of glutathione increased exponentially as incubation time increased (Fig. 2). The increase of glutathione content in the embryo, from 68 hours up to 140 hours must be due to transport of the yolk glutathione to the embryo, since there was no absolute increase in the whole egg. From this time on synthesis of glutathione

by the embryonic tissue started. These two separate phases are clearly seen on plotting time of incubation against the log of glutathione found in the embryo (Fig. 3).

Relation between glutathione and total nitrogen in the developing embryo.—It is known that total nitrogen increases in the embryo during incubation.

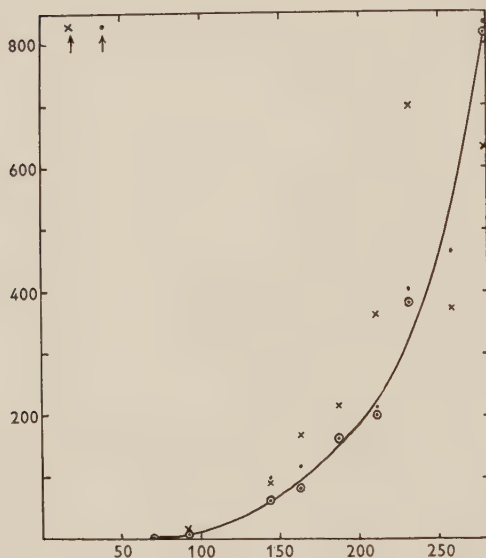


Fig. 2.—The glutathione content of chicken embryo. *Abcissa*, time of incubation in hours. *Ordinate*, glutathione in micrograms.

The increase in ribose nucleic acid also follows the increase of weight of the embryo, while desoxyribonucleic acid increase is much smaller [19, 24]. The glutathione content of the embryo seems to follow the total nitrogen content, and the ratio of total *N*:glutathione, with the exception of the data at 187 hours, appeared to be remarkably constant (Table II).

Glutathione reductase.—Glutathione in the chick embryo must be continuously oxidized as a result of keeping the numerous $-SH$ enzymes present in the cell in the reduced, active state. Yet, as no GSSG was found in the embryo, there must therefore be in the embryo glutathione reductase which keeps reducing any oxidized form. The presence of the enzyme was investigated in cell-free extracts of embryos from 72 to 235 hours of incubation. The embryos were cooled previously to $0^{\circ}C$ and were homogenized in the presence of $0.154\ M$ KCl. The suspension was immediately centrifuged in the Spinco ultracentrifuge for 30 minutes at 40,000 rpm. Since the supernatant solution contained glucose-6-phosphate dehydrogenase, the TPNH needed

for the reduction of GSSG was obtained by oxidation of glucose-6-phosphate by establishment of the coupled oxidation-reduction:

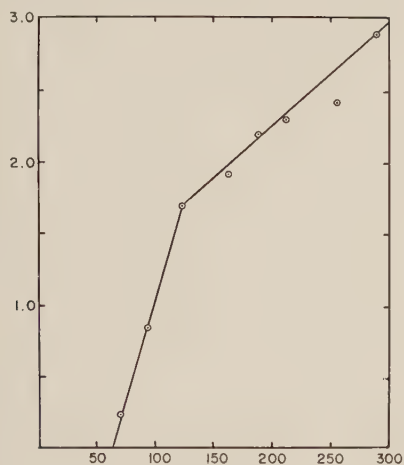
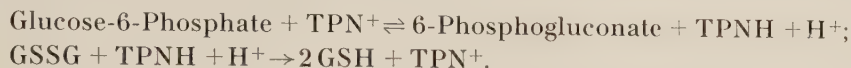


Fig. 3.

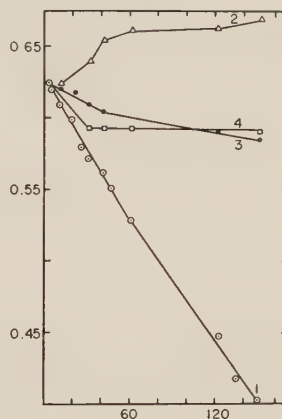


Fig. 4.

Fig. 3.—Rate of glutathione formation during development. *Abscissa*, time of incubation in hours; *Ordinate*, log. of glutathione concentration in micrograms.

Fig. 4.—Cystine reductase in the liver of 12-day chicken embryo (cell-free extract). *Abscissa*, time in minutes; *Ordinate*, O.D. at 340 mμ. 1, Cell-free extract, cystine, DPNH at time zero. 2, Cell-free extract, DPNH. 3, Cystine, DPNH. 4, Boiled cell-free extract, cystine, DPNH.

TABLE II. *Relation between total nitrogen and glutathione content in chicken embryo during development.*

Incubation h	Nitrogen μg	Glutathione μg	HSH per mg N μg
68-72	95	1.86	19.5
89-96	261	6.54	25.0
144	3,489	62.10	17.8
162-164	4,660	82.42	17.7
187	4,340	161.30	37.1
211	8,490	199.0	23.3
231	16,120	380.0	23.6
258	18,470	368.50	19.9
279	33,300	816.0	24.5

TABLE III. *Glutathione reductase in chicken embryo (cell-free extract) during development.*One unit = decrease in O.D. at 340 m μ of 0.0622/min/mg protein at 25°C.

Embryo incubation time h	Enzyme units
72	0.335
96	0.579
138	0.765
142	0.782
188	0.607
216	0.716
232	0.810

The enzyme system was as follows: glycylglycine buffer 0.1 M pH 7.5, 1 ml; TPN⁺, 1.5 μ moles, 0.5 ml; MgCl₂, 0.1 M, 0.3 ml; Versene, 0.075 M, 0.1 ml; glucose-6-phosphate, 0.4 μ moles, 0.1 ml; H₂O, 0.7 ml; cell-free extract, 0.2 ml. When reduction of TPN⁺ was completed (as followed spectrophotometrically in the Beckman DU spectrophotometer at 25°C), 0.1 ml of 0.1 M GSSG in 0.0375 M Versene was added, and the rate of oxidation of TPNH was followed by reading the optical density at 340 m μ . Glutathione reductase was found in the chicken embryo 72 hours after incubation, the earliest stage at which enough tissue could be collected for the measurement of enzyme activity. Enzyme activity increased markedly at 96 hours after incubation, and from then on increased slowly (Table III). The enzyme in chicken embryo was, like that of plants [24] and of rat liver [5], specific for TPN⁺, as DPNH was not active.

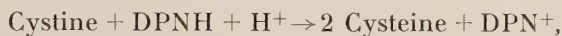
Cystine reductase.—Chicken embryo contained, in the liver, another -SS-reductase, cystine reductase. In contrast to glutathione reductase, this enzyme had as the oxidizing catalyst DPNH, TPNH being ineffective. Because of the slight solubility of cystine in neutral solution, the enzyme was assayed in Thunberg cells as follows: cystine, 12 μ moles in 0.1 M tris buffer, pH 8.7, 2.4 ml; cell-free extract, 0.5 ml; DPNH, 1.2 μ moles, 0.1 ml in the side bulb. The Thunberg cell was evacuated and filled with nitrogen. DPNH was added to the cell at zero time. There was steady oxidation, as shown by the decrease of the optical density at 340 m μ . There was some oxidation of DPNH on addition of cystine in the absence of the enzyme, but it was small compared to the enzymatic oxidation; the rate was similar to that found

TABLE IV. *Glucose-6-phosphate dehydrogenase (G-6-P Dehyd), aldolase-phosphoglyceraldehyde dehydrogenase (PGA Dehyd), and isocitric dehydrogenase in cell-free extracts of chicken embryo during development.*

System as described by Ghiretti and Barron [8]. Unit, increase in O.D. of 0.0622 at 340 m μ /min/mg protein at 25°C.

Incubation, h	G-6-P dehyd.	PGA dehyd.	Isoc. dehyd.
72	0.318	0.698	
96	0.650	0.639	0.718
120	0.396	0.679	0.626
144	0.450	—	—
192	0.435	—	—
210	0.456	0.480	—
240	0.525	0.610	1.793
264	0.303	0.472	1.130

with the boiled extract (Fig. 4). To make certain that oxidation of DPNH was due to reduction of cystine to cysteine according to the equation:



0.5 ml of 35 per cent trichloroacetic acid was added to the Thunberg cell, and after centrifugation cysteine was determined by the nitroprusside reaction. There was a net formation of 0.459 μ mole of cysteine, and 0.38 μ mole formation of DPN $^+$. (We cannot explain the discrepancy between the amount of cysteine formed and the amount of DPNH oxidized.) The enzyme found in embryonic chicken liver has therefore the same coenzyme specificity as the cystine reductase found in yeast by Romano and Nickerson [26].

Some enzymes for glucose metabolism.—The early claim of nonphosphorylating glycolysis in chicken embryo [18] was disproved by Meyerhof and Perdigon [15], who demonstrated the presence of the key enzymes for the glycolytic pathway of glucose. This finding has been confirmed [30, 20]. It was not known, however, whether there were enzymes for the phosphorylated oxidation pathway. It was our aim to study the time of appearance and the development of these enzymes during the growth of the embryo. Unfortunately the amount of tissue contained in embryos 24 and 48 hours old was so small that it was not feasible to prepare cell-free extracts. The extracts obtained from 3-day embryos contained the two main enzymes for the glycolytic and the phosphorylated oxidation pathways, as represented by aldolase-phosphoglyceraldehyde dehydrogenase, and by glucose-6-phosphate

TABLE V. *The metabolism of ribose-5-phosphate by homogenates of the liver of chicken embryo at different stages of development.*

Conditions: Homogenate in 0.154 M KCl, 1 ml; buffer, 2.7 ml; ribose-5-phosphate, 30 μ moles in 0.3 ml. Buffer, in aerobic experiments, Ringer-phosphate pH 7.4; gas phase, air. In anaerobic experiments, Ringer-bicarbonate, pH 7.4, gas phase, N₂: CO₂. Values give μ mole/mg dry weight/h. Temp. 38°C.

Incubation days	R-5-P utilization		Fructose formation	Heptulose formation
	Anaer.	Aerobic		
8	3.83	3.72	—	—
11	—	2.61	0.654	0.977
13	3.03	2.35	0.729	1.076
14	3.32	3.42	—	—
16	3.24	2.03	—	—
17	3.06	2.75	—	—
19	2.45	2.48	—	—
20	1.77	1.88	0.414	0.634

dehydrogenase. The activity of phosphoglyceraldehyde dehydrogenase was at its highest value as soon as it could be tested, in embryos three days old. The activity of glucose phosphate dehydrogenase doubled from the third to the fourth day. The activity of isocitric dehydrogenase increased greatly during the last days of embryonic development (Table IV).

The metabolism of ribose-5-phosphate.—The presence of glucose phosphate dehydrogenase in chicken embryo is an indication that the embryo might utilize the phosphorylated oxidation pathway. This pathway is not uncommon in tissues of rapid growth. Villavicencio and Barron [32] have shown that in tumors 30 per cent of the metabolism proceeds *via* phosphorylated oxidation. Ribose-5-phosphate was rapidly utilized by homogenates of the liver of chicken embryo, both aerobically and anaerobically. Under both conditions the rate of utilization, very high in the liver of eight-day embryos, diminished gradually with the growth of the embryo. That the metabolism of ribose-5-phosphate follows the pathway established by Horecker [8] and by Racker [28] is shown by the formation of fructose and of heptulose (Table V). The homogenates did not utilize ribose.

For comparison with its utilization in the liver of adult animals, the metabolism of ribose-5-phosphate was also studied in liver slices of adult chicken and of adult rat and pigeon. The utilization was highest in the liver of chicken embryo. Here, as in the homogenates, ribose-5-phosphate metabolism de-

TABLE VI. *Pentose metabolism of adult and embryonic liver (tissue slices).*

Slices suspended in Ringer-phosphate, pH 7.4. Gas phase, air. Temp. 38°C. Values give $\mu\text{mole}/\text{mg}/\text{dry weight}/\text{h}$. Initial amount of R-5-P: 30 μmoles (dry weight = 20–25 mg).

Animal species	R-5-P utilization	Fructose formation	Heptulose formation	Ribose utilization	Fructose formation
Adult					
Pigeon	0.224	0.040	0.045	—	—
Rat	0.246	0.063	—	0.042	0.003
Chicken	0.257	0.048	0.046	0.065	0.002
Embryo, chicken					
12 days	0.812	—	—	—	—
13	0.395	0.070	—	—	—
17	0.482	0.088	0.10	0.032	0.008
19	0.280	—	—	—	—

creased as the embryo grew. The utilization by the liver of the three adult animals—rat, chicken, and pigeon—was roughly similar, and less than in embryonic liver. There was a small utilization of ribose in the adult liver, and the liver of 17-day embryos (Table VI). It seems that ribokinase, which is absent in embryonic liver during the first days of development, appears a few days before maturity of the chick embryo.

DISCUSSION

The experiments presented here on the glutathione content in hen's eggs and in the chicken embryo during development have shown that in the egg, before incubation, there is a small amount of glutathione, and that synthesis of the tripeptide starts about six days after incubation when the liver becomes functionally active. There seems to be, however, a definite transfer of glutathione from the yolk to the embryo, as demonstrated by the continuous increase of glutathione in the embryo, while the total amount of glutathione in the whole egg remained approximately constant. The histochemical observations of Buño [2] are in agreement with this contention. Glutathione is required not only during the process of cell division, but also during cellular growth, as was observed by Hughes [10] on addition of iodoacetamide to tissue cultures of chick embryo. The synthesis of glutathione, as shown by the total increase, was parallel with the increase in total nitrogen and in

ribonucleic acid. All the glutathione in the chick embryo seems to be in the reduced form, since no GSSG was observed on paper chromatography of the ultrafiltrates. It may be argued that the addition of KCN reduced some of the GSSG present in the extracts [3]. However, no GSH spots were observed on addition of KCN to the standard GSSG. The glutathione reductase, which was present in the embryo from the time enzyme determinations were feasible, kept all the glutathione in the reduced state.

The liver of the chicken embryo contained also cystine reductase, which differed from glutathione reductase in that the specific coenzyme was DPNH instead of TPNH. Since the liver is the main source for protein synthesis, it is possible that the reductase is concerned with the production of cysteine necessary for the synthesis of the large number of -SH-containing proteins. We may venture to postulate that, while glutathione is needed by the embryonic tissue to increase the rate of synthetic reactions through its high reducing intensity, and to keep the -SH enzymes in the reduced, active state, cysteine is required for the synthesis of -SH proteins and other structural units containing this amino acid. The two reductases—glutathione and cystine reductase—thus play an important role in embryonic development.

The presence of the enzymes for phosphorylated oxidation and the rapid utilization of ribose-5-phosphate show that in chick embryo a large proportion of glucose is utilized by the phosphorylated oxidation pathway, as occurs in tumors, which are also rapidly growing tissues. As the embryo approached maturity the rate of ribose-5-phosphate utilization diminished until at the end of the incubation period, utilization had decreased to the rate found in the adult tissue.

SUMMARY

Glutathione was determined in hen's egg before and after incubation, and in chicken embryos during development. In the egg it was found only in the yolk, and the total amount remained constant up to six days after incubation. Synthesis started after this period and the amount increased rapidly. In the embryo the increase of glutathione was parallel to the increase of total nitrogen.

The embryonic liver of the chicken contained cystine reductase, an enzyme which reduced cystine to cysteine with DPNH as the specific coenzyme. TPNH was ineffective. Glutathione reductase, TPNH-specific, was also present.

Chicken embryo contained the enzymes for the glycolytic and the phosphorylated oxidation pathways of glucose metabolism from the time reliable determinations were feasible. The rate of utilization of ribose-5-phosphate in the early embryonic liver was three times as fast as that of the adult liver. The rate diminished as the age of the embryonic liver increased.

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THE EFFECTS OF TRYPSIN ON THE SIZE, VIABILITY AND DRY MASS OF SARCOMA 37 CELLS

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DURING the course of another investigation, it became necessary to evaluate the effects of trypsin on the size, viability and dry mass of Sarcoma 37 ascites cells. This communication deals with these experiments. As trypsin-dispersion of tissues is a common tissue-culture technique, the results may well be of general interest.

When tissues are treated with trypsin as, for instance, in the method described by Youngner [16], an amorphous glutinous material appears in which many dispersing cells become embedded. This glutinous material, which has been observed by the author after trypsinising many different types of human tumours, also appears when Sarcoma 37 cells are so treated.

MATERIALS AND METHODS

Preparation of cell suspensions and treatment with trypsin.—Trypsin (Difco 1:250) made up to 0.25 per cent in phosphate-buffered saline (pH 7.2) as described by Dulbecco [5] was used throughout. Sarcoma 37 ascitic fluid was obtained from mice of a heterozygous strain and 0.1 ml Heparin (B.D.H. 5000 U/ml) was added to each 3 ml of this fluid. The cells were then washed three times in phosphate-buffered saline at 4°C, by slow centrifugation (400-500 rpm). Similar dilute suspensions of the cells were made up in trypsin solution and in phosphate-buffered saline (control) in 10 × 75 mm test-tubes.

The tubes were incubated in a 37°C water-bath for 35 minutes. On being removed from the bath, the cells were again washed three times as described above. The tubes were then placed vertically in a beaker of melting ice for one hour, after which the top layer of fluid, which is a single-cell suspension, was pipetted off and placed in another tube in the ice. Samples were taken from this tube as required. This sedimentation technique has been fully described by Hewitt [9], and is known to yield representative samples of free tumour cells.

Measurement of cell diameters.—The cells were placed in a Fuchs-Rosenthal chamber and their diameters were measured photographically through a × 8 eyepiece and the × 40 objective of a phase-contrast microscope. Some thousands of cell diameters have been measured.

Viability tests.—Viability of the control and trypsinised cells was assessed by three methods—

(1) Differential counts of cell suspensions viewed by phase-contrast microscopy. The cells with regular, well-defined edges and clear halos were taken as being alive; the others were considered degenerate.

(2) The trypan-blue take-up technique, as originally described by Pappenheimer [11].

(3) Animal titrations as described by Hewitt [9] using a T.D. 50 technique. Heterozygous albino mice were given accurately measured subcutaneous injections of 0.2 ml of cell suspension containing a mean of 2000 Sarcoma 37 cells. Each mouse was injected in both axillae and both groins. The experiment was performed on 20 mice, i.e. 40 injections of trypsinised, and 40 injections of control cells were given. The mice were examined periodically for takes and killed and re-examined after 15 days.

Determination of dry mass.—It has been shown by Davies *et al.* [4] and Barer *et al.* [1] that the dry mass m , of a cell of area A square centimetres, may be expressed by the formula

$$m = \frac{\theta A}{100 \alpha}$$

when the cell is immersed in water; θ is the optical path difference through the cell and α is the mean refractive increment of the intracellular contents. This value for α may be taken as 0.0018 for Sarcoma 37 cells.

If a cell of thickness t cm is immersed in a fluid of refractive index μ_m , then

$$m = \frac{\theta A}{100 \alpha} + (\mu_m - \mu_w) \frac{A t}{100 \alpha},$$

where μ_w is the refractive index of water [4].

The refractive indices of the suspending fluids of the trypsinised and control cells after washing, were found to be the same, 1.3403 at 20°C.

In the previous two experiments no change had been noted in the numbers of dead cells present or in the frequency distribution curves of mean cell diameters, between the trypsinised and control cells. It was thus assumed that the dry mass of individual cells of the same diameter in both groups was proportional to the optical path difference of the light going through them.

With the Baker interference microscope, the optical path difference θ is calculated from the rotation of the goniometer in degrees.

$$\theta = \frac{\text{Goniometer reading} \times 2}{360} \times \text{wavelength of light used.}$$

Thus, with cells of the same diameter in the same immersion fluid, the goniometer reading is proportional to the optical path difference which in turn is proportional to the dry mass of the cell.

Dry mass determinations were made on the Baker interference microscope fitted with the half-shade eyepiece described by Smith [13]. Cell suspensions

were examined in ringed vaselined slides, through No. 1 coverslips. The $\times 100$ water-immersion shearing objective was used throughout. A $\times 10$ eyepiece fitted with a micrometer was used to select cells of the same diameter, and a "Projectolux" base with a Didymium Green filter (maximum transmission 5500 Å) provided the illumination.

Goniometer readings were taken as nearly as possible through the centre of the nucleus, where the reading was greatest. As many cells as possible of the same diameter were measured in ten-minute periods, and fresh preparations were made from tubes stored in melting ice.

RESULTS

Cell diameters.—No significant difference in mean cell diameters (15.8μ ; standard deviation = 3.8) was observed between the trypsinised cells and the control series, nor was there any difference in the frequency distribution between cells taken straight from the mouse and suspended in ascitic plasma, and those incubated in the water-bath as described.

Viability.—By phase-contrast and Trypan Blue methods, it was shown that in both trypsinised and control cells the number of viable cells present was 87–88 per cent. There were takes in 17/40 sites injected with the control series, and 18/40 in those injected with the trypsinised cells. Thus there was no appreciable change in viability due to trypsin *per se*.

Dry mass.—500 cells were measured in each group. The frequency distribution was normal in each case and the coefficient of variation ranged from 14.2 to 16.9. The difference between the mean goniometer readings for the two groups of cells corresponds to a loss in dry mass, after treatment with trypsin, of the order of 20 per cent.

DISCUSSION

The results of the experiments show that no change in viability or mean cell diameter occurs when Sarcoma 37 cells incubated in phosphate buffered saline are compared with those incubated in a similar solution containing 0.25 per cent trypsin. There is, however a loss of dry mass of some 20 per cent due to the trypsin, and this is not associated with loss of viability.

A possible explanation for the loss of mass is that trypsin treatment liberates some mucinous capsular material, as occurs with the spore coats of *B. megatherium* and other organisms [14]. Against this hypothesis is the fact no extracellular coat is evident in electron micrographs of these cells [6] and, if such a coat were present and consisted of mucopolysaccharide material,

it would be very obvious in electron micrographs [7]. Moreover, attempts to stain such a coat by Hale's method [8] which utilises the formation of a complex between dialysed iron and certain mucopolysaccharides, have been unsuccessful.

Whatever the weight loss is due to, it would appear to be additional to the "handling" loss demonstrated by Puck *et al.* [12] with radioisotopes, as this traumatic loss would have occurred in the control series as well as in trypsinised cells.

These initial observations have been confined to Sarcoma 37 cells on account of their regular, spherical shape, this feature being essential if the optical path difference of large numbers of cells is to be measured rapidly by visual interferometry. Cells of irregular shape could be measured however, by means of integrators described by Mitchison *et al.* [10] and Davies *et al.* [3], and the scanning devices described by Caspersson *et al.* [2] and Svensson [15].

Further experiments to identify and localise this weight loss are being made.

SUMMARY

Treatment of Sarcoma 37 ascites cells with trypsin was found to be associated with a significant loss of dry mass which was unaccompanied by any appreciable change in the mean cell diameter or the viability of the cells.

Throughout these experiments I have received considerable help and encouragement from Professor R. J. V. Pulvertaft and Dr. H. B. Hewitt.

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FREE AMINO ACID CHANGES DURING CLEAVAGE IN *XENOPUS LAEVIS* EMBRYOS

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IN a previous study [8] the concentrations of free amino acids were measured in whole embryos and in parts of embryos and larvae of *Xenopus laevis*. Some free amino acids that were present in particularly high concentrations in certain larval tissues also appeared in high concentrations in the embryonic primordia of these tissues. It was therefore argued that the varying concentrations of free amino acids in different parts of the embryo were signs of tissue differentiation at the chemical level. But it was impossible to put forward any interpretation of free amino acid changes in the embryo as a whole, from morula to hatching stage, since during this period such a complex of developmental processes including cell division, morphogenetic movements and differentiation into several tissue primordia, is taking place. Moreover, the embryonic stages selected for analysis were widely enough separated for a multiplicity of morphogenetic events, possibly including fluctuations in amino acid concentration (cf. [9]) to have occurred between one stage and the next.

During cleavage, however, a much closer series of stages can be distinguished externally, and neither morphogenetic movements nor tissue differentiation occur. The interpretation of free amino acid changes in the whole embryo during cleavage might therefore present fewer difficulties than at later stages. Kutsky *et al.* [10] and Chen [6] give a few data for cleavage stages of *Rana* and *Triturus* respectively. They agree in observing a decrease in the concentration of free aspartic acid during cleavage, but whereas Kutsky *et al.* noted increases in free glutamic acid from 1-cell to 2-cell and from 2-cell to blastula stages, Chen found decreases in the glutamic acid, and increases in glutamine, from 2-cell to morula and from morula to early gastrula stages.

EXPERIMENTAL

In the present work a close series of pre-gastrulation stages of *Xenopus* embryos has been collected, and the changes in concentration of free aspartic acid, glutamic acid and glutamine have been measured by paper chromatography. These three

amino acids were chosen not only in the hope of elucidating the apparent disagreement between Kutsky *et al.*'s and Chen's results, but also because they are present in high enough concentrations to be measured in samples of as few as twenty embryos. It was found, however, that even during the collection of twenty embryos one further cleavage might occur in some of them, so that the stages could not be defined more exactly than: 2-4 cells, 8-16 cells, 32-64 cells and blastula (stage 8 of Nieuwkoop and Faber [13]). No data were obtained on uncleaved eggs since it was not possible to distinguish fertilised from unfertilised ones, and the infertile eggs often underwent a slow cytolysis which would result in abnormally high concentrations of free amino acids. All samples were placed in the deep freeze (-20°C) immediately after collection and subsequently frozen-dried, then weighed in the frozen-dried state. The procedure for extracting free amino acid and preparing chromatograms was as previously described [8] except that 80 per cent methanol was used as the extractant, and the propanol run was made first, followed by a run in water-saturated phenol without added ammonia or sodium cyanide. This procedure gave cleaner chromatograms than previously. For quantitative estimation of the amino acids Benz's modification [3] of Bode *et al.*'s procedure [4] was used.

RESULTS

The results (Fig. 1) show a definite fall in concentration of free aspartic acid ($p = < 0.01$), in agreement with the previous authors' findings in *Rana* and *Triturus*, but here rather more marked, dropping to half in the interval between the 2-4 cell stage and the blastula. During this time the concentration of glutamine is doubled—again a rather more marked change than was found by Chen in *Triturus* ($p < 0.01$ for the increase from 2-4 cell stage to 32-64 cell stage). In agreement with Kutsky *et al.* but in contradiction to Chen, the glutamic acid concentration increases during cleavage. The increase is not continuous, however, and between 2-4 cell and 32-64 cell stages is statistically significant only at the 5 per cent level. From 2-4 cell stage to blastula stage the increase is significant at the 2 per cent level. Changes of this kind could have passed undetected in Chen's measurements which were made on slightly different stages and on smaller numbers of samples than these. The simultaneous increase of both glutamic acid and glutamine found here does not rule out the possibility suggested by Chen that glutamine is formed at the expense of glutamic acid, but more definite evidence is needed to establish this point.

Studies with radioactively-tagged glutamine and glutamic acid (see [2, 12, 14]) indicate that they play a significant role as nitrogen donors during the synthesis of proteins and nucleic acids. Since the amphibian embryo has no external source of nitrogen, the increases of free glutamic acid and glutamine observed here must have resulted either from breakdown of

pre-existing protein or peptides, or from transformation of other free amino acids. It seems most reasonable to consider that the accumulation of these two amino acids during cleavage is an intermediate step in the mobilisation of nitrogen from storage reserves such as the yolk, and that this nitrogen will later be incorporated into new proteins synthesised as the embryo develops [7]. Glutamine may also participate in the synthesis of urea [1], since small quantities of urea are excreted during cleavage in *Rana* [5].

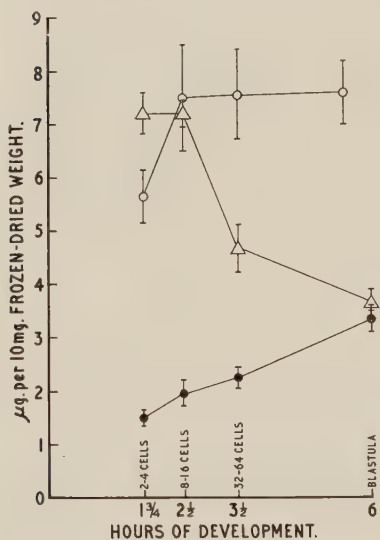


Fig. 1.—Concentrations of free amino acids during cleavage in *Xenopus* embryos. Each point is mean of 7 determinations. Vertical lines indicate ± 1 standard error of the mean. O, glutamic acid; Δ , aspartic acid; ●, glutamine.

It is somewhat surprising that aspartic acid should show an exactly opposite trend in concentration to glutamic acid, since these two amino acids play similar roles in nitrogen transfer. It has previously been suggested [8] that changes in concentration of aspartic acid in the embryo may be related to turnover of nucleoprotein, since nitrogen is known to be incorporated readily from aspartic acid into nucleotides [11]. Aspartic acid differs from other free amino acids (glutamic acid, glutamine, glycine, α -alanine, valine and leucine) in being more concentrated per unit dry weight in the animal half of the blastula than in the vegetal half [8]. Calculated *per cell*, however, its concentration is lowest in the animal half. This may indicate that more aspartic acid per cell has been utilized in exchanges with nucleotides in the animal half than in the vegetal half, owing to the greater number of divisions that the nuclei of the animal half have undergone. On this argument, the decrease in aspartic acid in the whole embryo during cleavage might also be explained as resulting at least partly from exchanges with nucleotides during

nuclear division. Some free aspartic acid may also be incorporated into cytoplasmic proteins. Definite conclusions about the fates of any free amino acids in the embryo must, however, await more conclusive experiments with tracer techniques.

SUMMARY

During cleavage stages in *Xenopus laevis* the concentrations of free glutamic acid and glutamine increase, while that of free aspartic acid decreases. It is argued that glutamic acid and glutamine may accumulate as intermediates during the mobilisation of nitrogen from storage reserves, while the decrease in aspartic acid may be due to exchanges with nucleotides.

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THE ACTIVITY OF MICROSOMES FROM REGENERATING RAT LIVER IN AMINO ACID INCORPORATING SYSTEMS

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As a response to partial hepatectomy a condition of rapid, compensatory growth is induced in rat liver. It is known from earlier studies that this condition is accompanied by an increased incorporation *in vivo* of labeled amino acids into the liver proteins [7, 8]. The increased incorporation is not merely due to an intensified uptake of extraneous amino acids into the liver cells, but rather to an activation of the intracellular incorporation mechanism proper. It was shown in a previous paper [15] that cell-free cytoplasmic systems isolated from regenerating rat liver had a considerably higher capacity for amino acid incorporation than systems of a similar kind prepared from normal liver tissue. The cytoplasmic systems used in these incorporation experiments contained soluble cell constituents in combination with microsomal material. It has been established by recent studies that in systems of this kind amino acid incorporation proceeds by way of a two-step reaction mechanism [9, 27, 14, 10]. Soluble enzymes from the cell fluid fraction, depending on adenosine triphosphate (ATP) for energy supply, are required for the initial activation of the labeled amino acids. The activated amino acids may subsequently be introduced into protein molecules by constituents of the microsomal material.

It was shown previously [15] that both of these reaction systems work with increased activity in cytoplasmic preparations from regenerating livers. The present experiments deal with the activation of the microsomal system. They show that the activation of this system takes place fairly rapidly after an initial lag period of about 12-14 hours. The activation is connected with a change in the composition of the microsomal material. On a protein or nitrogen basis the content of ribonucleic acid (RNA) in this material increases (cf. [22]) while the content of glucose-6-phosphatase, a microsome-specific enzyme [5, 6], decreases. It is known that the microsomal fraction mainly emanates from the endoplasmic reticulum, a submicroscopic structure composed of closely interrelated lamellar and granular

elements. Since the granules are particularly rich in ribonucleoproteins [21] while glucose-6-phosphatase, like certain other microsome-bound enzymes, is concentrated to the membranes (cf. [13, 21]), the experiments suggest that the proportion between these structural elements is changed during the regeneration period.

EXPERIMENTAL

Male albino rats weighing approximately 250 g were used. Each experiment included one control rat (sham operated) and 2–3 hepatectomized rats, from which the medial liver lobe, the left lateral lobe, and the posterior lobule of the right lateral lobe had been removed under ether anesthesia. Before the incorporation experiments the rats had been kept without food for 15–20 hours [13].

At different periods after operation (7 hours to 5 days) the animals were killed by decapitation. The livers were rapidly excised and minced in an ice-cold medium containing 0.25 *M* sucrose, 0.025 *M* KCl, 0.01 *M* MgCl₂, 0.03 *M* tris(hydroxymethyl)-aminomethane, and 0.02 *M* potassium phosphate. The pH of the medium was 7.8. After repeated washings the liver mince was ground in a glass-homogenizer with 2–2.5 volumes of the same medium. The homogenates were centrifuged for 5 minutes at 12,000 × *g* (0°C), and the upper part of the supernates (11 ml) was transferred to the tubes of a Spinco preparative ultracentrifuge. The lipid layers, which accumulated at the surface of the supernates, particularly in the case of the regenerating livers, were avoided. The mitochondria-free homogenates were centrifuged at 105,000 × *g* for 45 minutes, and the clear supernates were collected ("cell fluid fraction"). The microsome pellets were drained and repeatedly washed with the homogenization medium. A small, fluffy layer, which covered the translucent microsome pellets was completely removed by these washings. To each tube 2.5 ml of medium was added, and the microsome sediments were dispersed by homogenization.

The incubation samples contained in final volumes of 1.1 ml 0.65 ml of the cell fluid fraction from the normal liver, 0.08, 0.15 or 0.3 ml of the suspended microsomes from the normal or regenerating livers, 10 μ*M* of phosphoenolpyruvate (synthesized from epichlorohydrin [2]), 1 μ*M* of ATP (Sigma), and 0.08 μ*M* of C¹⁴-L-leucine (1.5 mc per mM). The samples were incubated at 35°C for 15 minutes.

After the incubations unlabeled L-leucine was added in large excess for isotope dilution, and the proteins were precipitated with trichloroacetic acid. In order to obtain the same protein content in all samples a supplementary addition of microsomes was made to those samples which had been incubated with the lower concentrations of microsomes. The proteins were purified by a modified Schneider technique [23] and plated. The specific activities of the samples were calculated for indefinite sample thickness (1 cm² plate area) on the basis of an empirical activity saturation curve.

The following analyses were carried out on diluted samples of the microsomal suspensions: total nitrogen, protein, RNA, and glucose-6-phosphatase. The protein content was determined by the method of Lowry *et al.* [19], crystallized bovine plasma albumin (Armour Lab.) being used as a standard. For RNA-analyses 0.5 ml samples were hydrolyzed with 2.0 ml of 0.2 *M* HCl at 90°C for 17.5 minutes. The

TABLE I. *Incorporation of C¹⁴-L-leucine into proteins by the rat liver system used in the experiments. Characteristic requirements for the isotope incorporation.*

The complete system contained 0.65 ml of cell fluid, 0.2 ml of a standard suspension of microsomes, 0.08 μ M of C¹⁴-L-leucine (1.5 mc per mM), 10 μ M of phosphoenolpyruvate (PEP), and 1 μ M of ATP, and it was buffered at pH 7.8. The final volume was brought to 1.1 ml by the addition of homogenisation medium. Incubation for 15 minutes at 35°C.

	C.p.m. per cm ²
Complete system	300
minus cell fluid ^a	14
minus microsomes ^a	5
minus PEP	9

^a To give equal amounts of protein in all samples the omitted cytoplasmic component was added after the incubation.

hydrolysates were chilled in ice, and 0.06 ml of 70 per cent perchloric acid was added. After centrifugation the absorption curves of the extracts were recorded. Samples extracted with cold 0.2 M perchloric acid were used as blanks. The extinction at 260 m μ was read against an RNA-standard. Determined by this method the average concentration of RNA in the normal microsomes was 0.82 mg per mg nitrogen (cf. [21, 25]). Glucose-6-phosphatase activity was determined by the method of de Duve *et al.* [5].

The contents of total nitrogen and protein were determined in the cell fluid fractions also.

The rather simple fractionation and washing procedures used in the experiments gave a fairly acceptable separation between the microsomes and the activating enzymes in the cell fluid fraction (Table I). The effects which were obtained with the microsomal preparations were therefore ascribed to the microsomal constituents proper.

RESULTS

As was described in the experimental section microsomal suspensions were prepared in parallel from normal and regenerating livers and were tested simultaneously in incorporation systems containing standard amounts of normal cell fluid. It seemed desirable to perform these comparisons on the basis of equivalent microsomal concentrations. For practical reasons, however, it was not possible to carry out the analyses of the microsomal suspensions before the start of the incorporation experiments. In order to attain adequate conditions of comparison between the two kinds of microsomal preparations they were therefore added to the incubation samples

in series of increasing concentrations. The incorporation values obtained were afterwards plotted against the actual microsomal concentrations on the basis of the analytical data (total nitrogen, protein, RNA and glucose-6-phosphatase activity).

A typical experiment is illustrated by Fig. 1. This experiment was performed 16½ hours after the partial hepatectomy. As is shown by the figure, no significant incorporation was observed, when the system was incubated in the absence of microsomes. The incorporation increased gradually with increasing microsomal concentrations. By comparing the incorporation curves for the regenerating and normal microsomes at equivalent microsomal concentrations the relative activation effect was calculated. As is illustrated by Fig. 1, the measure of the activation varied, however, with the different kinds of analytical data by which the microsomal concentrations were determined. When calculated on the basis of the nitrogen content the activation quotients were slightly lower than on the basis of the protein concentration. Considerably much lower activation values were obtained, however, when the RNA-content was used as a standard (Fig. 1*b*). On the other hand, the relative activation effects were markedly increased when the incorporation curves were based on the activity of glucose-6-phosphatase (Fig. 1*c*).

The altered composition of the microsomal suspensions during the regeneration period, which was responsible for these divergencies, is more fully illustrated by Fig. 2. On the basis of equal protein content the amounts of RNA began to rise about 12–14 hours after the hepatectomy, while instead the glucose-6-phosphate activity simultaneously decreased.

The relative activity of the microsomes in the incorporation systems showed a corresponding rapid increase in regenerating livers after an initial lag period of 12–14 hours. The time course of the activation process is illustrated by Figs. 3 and 4. In Fig. 3 the relative microsomal activities of the regenerating livers have been calculated on the basis of equivalent concentrations of protein in the microsomal suspensions. The values shown in Fig. 4 were obtained on the basis of equal glucose-6-phosphatase activities and RNA-contents respectively. The experiments suggest that full microsomal activation was normally reached about 30 hours after the hepatectomy.

DISCUSSION

It was shown in a previous report [15] that microsomal fractions, which had been prepared from regenerating rat liver, worked more effectively in amino

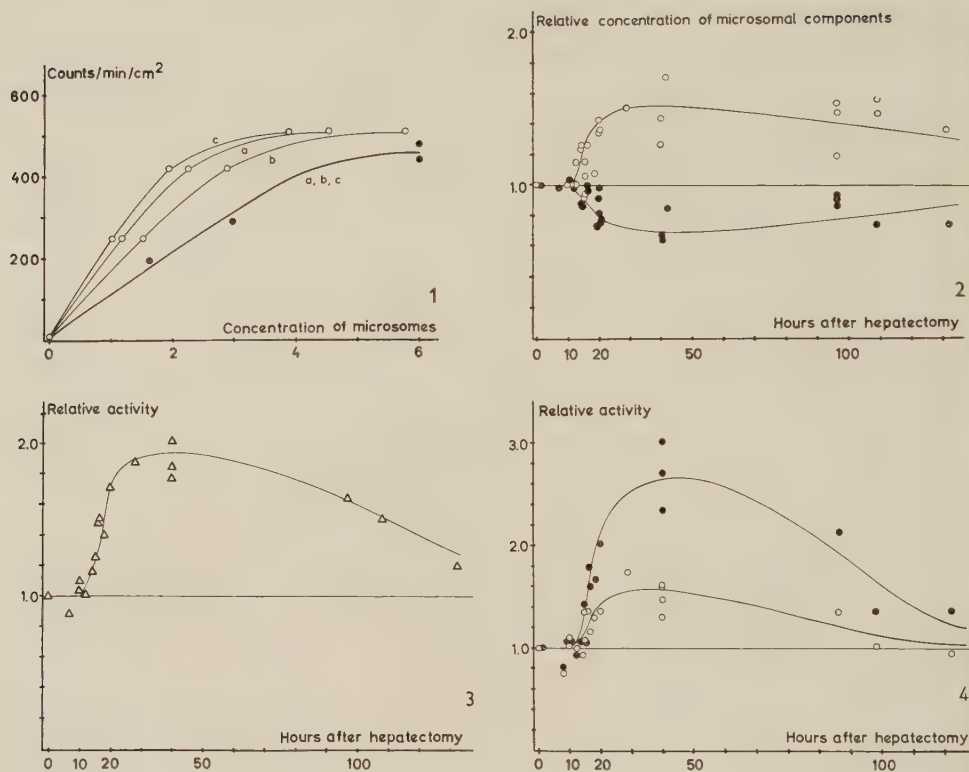


Fig. 1.—The incorporation system described in Table I adapted to the determination of microsomal activation. The activities of microsomes from normal (●) and regenerating (○) livers, 16½ hours after partial hepatectomy. The incorporation curves are plotted on the basis of equivalent protein content (a), RNA-content (b) and glucose-6-phosphatase activity (c) in the two kinds of microsomal samples. Each unit at the horizontal axis represents: (a) 1 mg of protein (determined by the method of Lowry *et al.* [19], bovine albumin being used as the protein standard), (b) 0.18 mg of RNA, (c) a glucose-6-phosphatase activity of 2.2 μ M of hydrolyzed phosphate per 10 minutes at 35°C.

Fig. 2.—The effect of partial hepatectomy on the composition of microsomal fractions. The microsomes from regenerating livers are compared with normal microsomes on the basis of equal protein content. Relative RNA-content (○) and glucose-6-phosphatase activity (●) of microsomes from regenerating livers at different periods after hepatectomy. The analytical values obtained from normal microsomes are symbolized by the horizontal line.

Fig. 3.—The activity of microsomes for amino acid incorporation at different periods after partial hepatectomy, determined by the method illustrated by Fig. 1. The microsomes from regenerating livers are compared with normal microsomes on the basis of equal protein content. The incorporation values obtained with normal microsomes in each experiment are indicated by the horizontal line.

Fig. 4.—The activity of microsomes for amino acid incorporation at different periods after partial hepatectomy. Activation values represented as in Fig. 3, but on the basis of equal glucose-6-phosphatase activity (●) or RNA-content (○).

acid incorporation systems than similar preparations from normal liver. The microsomal suspensions used in these experiments were standardized on the basis of equal liver weights. The experiments did not decide, therefore, whether the effect was due to an increased proportion of microsomal material in the regenerating liver tissue or to a specifically higher activity of such microsomes in the incorporation system.

The experiments described here were in part planned to elucidate this question. In the same way as in the previous experiments the microsomal activities were measured by means of a common amino acid activating system containing normal cell fluid. It is believed that under these conditions the supply of activated amino acid available to the two parallel sets of microsomes was nearly identical, and that any isotope dilution, effected by endogenous amino acid, may have been similar. The microsomal activities were compared on the basis of equal microsomal concentrations, determined by analyses of certain microsomal components.

As would be expected in view of the well-known variability of the regeneration process, the data obtained were subject to a considerable scattering (Figs. 2, 3, 4). As a matter of fact, growth examination at different times after the hepatectomy showed variation in the apparent regeneration by a factor of several hours. The most characteristic feature of the time course of the microsomal activation was the drastic rise in the relative incorporation capacity about 15–20 hours after the hepatectomy. Simultaneously with this rise there was an increase of the RNA-concentration in the microsomal fractions. On the contrary, the activity of glucose-6-phosphatase decreased.

It has recently been shown by electron microscopy that increased amounts of ergastoplasmic aggregates appear in the cytoplasm of rat liver 18–20 hours after partial hepatectomy [4]. As has been pointed out by Palade [20] the endoplasmic reticulum in liver cells is locally differentiated. By electron microscopy two types of reticulum may be recognized, one type with smooth surfaced profiles, and one type with rough surfaced profiles, covered with attached nucleoprotein particles [21]. A whole spectrum of intermediary forms exists between these two types. The rough surfaced elements correspond to the ergastoplasmic structures just mentioned.

In view of these observations the following interpretation of the experiments may be suggested. After a lag period of 12–14 hours a formation of ribonucleoprotein particles is induced in hepatectomized rat liver, evidenced by the increased proportion of RNA in the microsomal fraction. In all probability, the granules are attached to the membranes of the endoplasmic reticulum, but there is no information available at present, whether they are

formed in connection with this structure or originate from elsewhere, viz. the nucleus (cf. [1, 3]), the factors regulating the induction process being entirely unknown. Since glucose-6-phosphatase apparently is tied up with the lamellar components of the microsomal fractions (cf. [13, 21]), the altered proportions between the activity of this enzyme and the RNA-content in the microsomal fraction during regeneration may indicate a changed proportion between the lamellar constituents of the reticulum and the nucleoprotein granules (Fig. 2). It is not known at present, to what extent the activity of glucose-6-phosphatase is correlated with the quantity of cytoplasmic lamellae in the liver cells. The fact that the activity of this enzyme per average cell is approximately constant during regeneration [26] suggests, however, that the amounts of lamellar material is not substantially changed during the induction period. Ergastoplasmic regions of the reticulum may be more closely filled with granules, and smooth areas (in the sense of Palade) may gradually be transformed into ergastoplasm. Since it is the nucleoprotein components of the structures which are primarily responsible for the amino acid incorporation [11, 12, 18, 17], fragments of the reticulum, prepared in the form of microsomes, would become relatively more active in incorporation experiments after this modification.

As is shown by Figs. 2 and 3 the activation of the microsomal material was proportionally higher than the increase of the RNA-content. This fact may indicate that the capacity for amino acid incorporation is determined not exclusively by the nucleoprotein constituents as such, but to some extent by secondary regulation factors operating in the material. An alternative explanation of this disproportion may be suggested, however. Since magnesium ions were included in the homogenization medium, soluble nucleoproteins may be precipitated by this agent in the form of microsome-like units [12, 24]. In all probability, such precipitation particles would be inactive in the incorporation system, but they would influence the RNA-analyses. If it is assumed that the soluble nucleoprotein fraction were less effected by the hepatectomy than the microsomal nucleoproteins, the actual increase of the latter fraction would consequently be underestimated.

It was mentioned in a previous paper [15] that also the amino acid activating enzymes of the cell fluid fraction becomes activated during the regeneration cycle. A study of the activation process, based on incorporation experiments and on measurements of pyrophosphate exchange, will be published.

SUMMARY

Microsomal suspensions were prepared in parallel from normal and regenerating rat livers, and their activity for amino acid incorporation was determined in incorporation systems containing C^{14} -L-leucine as the labeled component and standard amounts of normal cell fluid as the source of activating enzymes. The concentrations of the microsomal suspensions were determined by analyses of total nitrogen, protein, ribonucleic acid (RNA) and glucose-6-phosphatase.

When the relative microsomal activities for amino acid incorporation were compared at different periods after hepatectomy a rapid rise was observed after a lag period of about 12–14 hours. This rise occurred simultaneously with a change in the composition of the microsomal material. On the basis of equal protein concentrations the amounts of RNA increased while the activity of glucose-6-phosphatase decreased. The change may indicate modified proportions between the granular and lamellar constituents of the endoplasmic reticulum, from which the microsomal material emanates. The increased microsomal activities during the regeneration period may be conditioned by a higher average concentration of nucleoprotein granules connected with the reticular membranes.

An account of this investigation was presented at the Conference on Cell Research, Stockholm, 28 February–2 March, 1957 [16].

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A SIMPLE PLASTIC PERFUSION CHAMBER FOR CONTINUOUS MAINTENANCE AND CINEMATOGRAPHY OF TISSUE CULTURES¹

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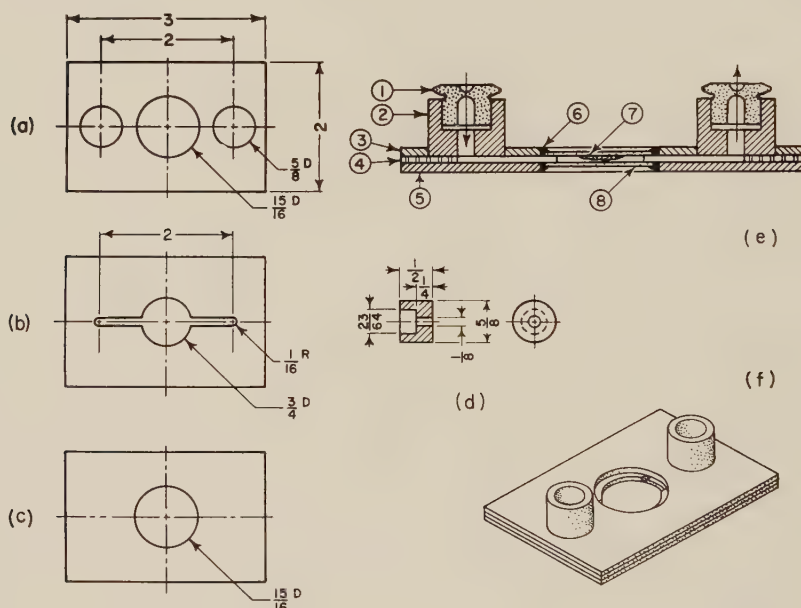
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SINCE the earliest attempts to maintain living tissues *in vitro*, it has appeared desirable to construct culture chambers in such a manner that the nutrient medium might be altered, exchanged, or continuously perfused without inadvertent disturbance of the contained explant. This ideal, as yet, has not been accomplished. Burrows, in 1912, devised a chamber of cork with glass windows, perfusate being conducted through the system along a frayed wick [6]; shortly thereafter, an all-glass version of the same apparatus was published by Romeis [21]. Various types of closed flasks have been suggested for long-term culture techniques, but have not permitted simultaneous microscopic observation [8, 10, 16]. Several glass chambers, with the tissues mounted on removable coverslips, have been suggested by de Haan and his co-workers [12, 13, 15]. Later designs have proved more practicable for direct photomicrography; generally constituting a central frame or core, with perfusion ports and coverslips, the chambers have been fabricated entirely of glass [23, 25, 27], or glass parts locked with metal clamps [11], and, more recently, of stainless steel frames of varying complexity [5, 24, 26]. A simple perfusion chamber, reported by Pomerat and his colleagues, consisting of a machined aluminum or stainless steel frame supporting a tissue compartment built up with coverslips and wax, has achieved some popularity [14, 17, 18]. Several contemporary designs have involved a core of rubber through which needles might be inserted, coverslips, and demountable stainless steel plates clamped by screws [19, 22].

The Pomerat chamber [14, 18] had been employed in this laboratory, but although optically satisfactory, was found inconvenient and time-consuming in assembly and manipulation. In the search for a desirable substitute, certain minimal requirements were established:

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- (1) Simple design and inexpensive fabrication, approaching the ideal of a single-shot, disposable unit.
- (2) Rapid loading by minimally skilled personnel.
- (3) Convenient exchange or perfusion of medium.
- (4) Freedom from toxicity or contamination.
- (5) Simultaneous microscopic observation and photography, with a short optical path for phase microscopy.
- (6) Easy demounting, cleaning, and sterilization.



a resin claimed to have properties ideal for the purpose [2, 3], and the same material, in fact, has provided the body of a demountable perfusion chamber for continuous culture [4]. From the standpoint of convenience, perhaps the most attractive design yet proposed for a plastic material has been that of Christiansen *et al.*, in which the frame has been cut from a single piece of Plexiglas, save for two inlet tubes cemented to it, and planned to fit a microscope stage and provide an optical path length of one millimeter [9].

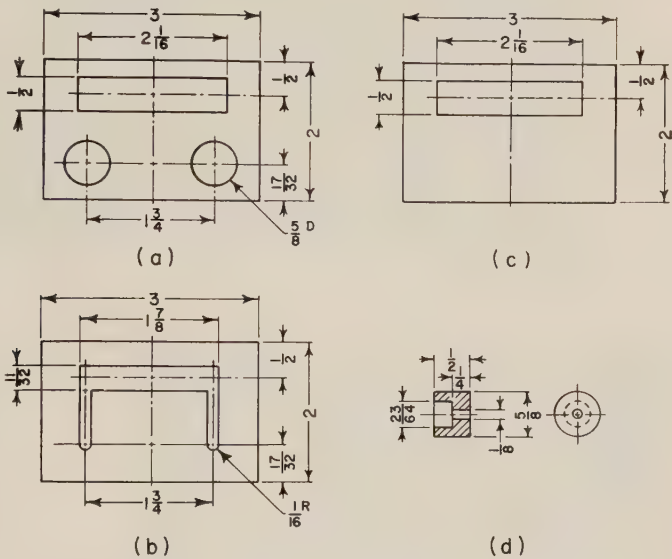


Fig. 2.—Design for a chamber to accommodate 11×50 millimeter coverslips. All dimensions in inches. (a) Roof plate; (b) core plate; (c) base plate; (d) duct (two required).

To afford broad machining tolerances with least sacrifice of optical accuracy, a simple laminated design has been prepared in this laboratory. The chamber proper consists of three pieces: (1) a base plate with an opening for the lower window; (2) a core plate serving as a support for the coverslips; (3) a roof plate with openings for the upper window and the two duct tubes through which medium is perfused. The ducts are cemented in place. Tissues may be mounted directly on either window, a standard coverslip fastened to the chamber by a paraffin-beeswax mixture. The basic design may be varied somewhat to accommodate various types of coverslips; three representative models are shown in the figures.

The earliest samples were made from nylon, which, while resisting autoclave sterilization without damage, could be cemented only with solutions that were directly toxic to tissues. It was claimed by Barski that Araldite was free of certain toxic effects present in Plexiglas [3]. In this laboratory,

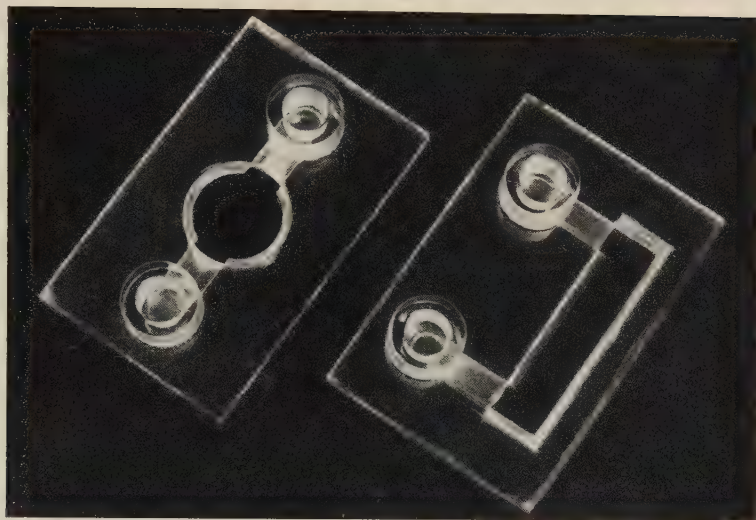


Fig. 4.—Plastic chamber frames designed for $\frac{7}{8}$ -inch diameter circular coverslips, at left, and 11×50 mm rectangular coverslips, at right.

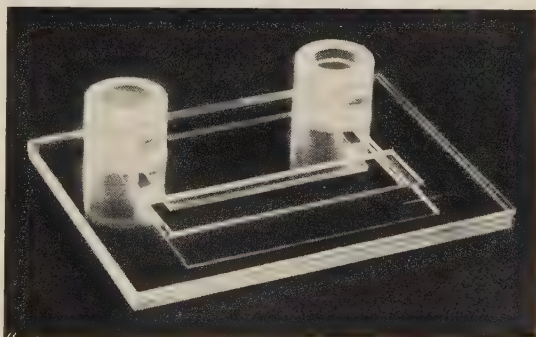


Fig. 5.—Chamber frame to accommodate 11×50 mm and 43×50 mm coverslips. Compare with working drawings in Fig. 3.

dissolved resin; then the roof, core, and base plates are clamped together under pressure until the primary set has taken place. In a few minutes the two duct tubes may be cemented to the frame. After overnight storage in an ordinary incubator to assure volatilization of superfluous solvent, the chamber is polished and cleaned.

Autoclave sterilization has not been used because of the risk of deformation, but might be permissible with suitable precautions. Roentgenological sterilization has been found convenient and effective; after the clean chamber is wrapped in paper or cloth, it is irradiated for 15 seconds with a short-cone dental x-ray set at 65 kilovolts. Equally satisfactory results may be

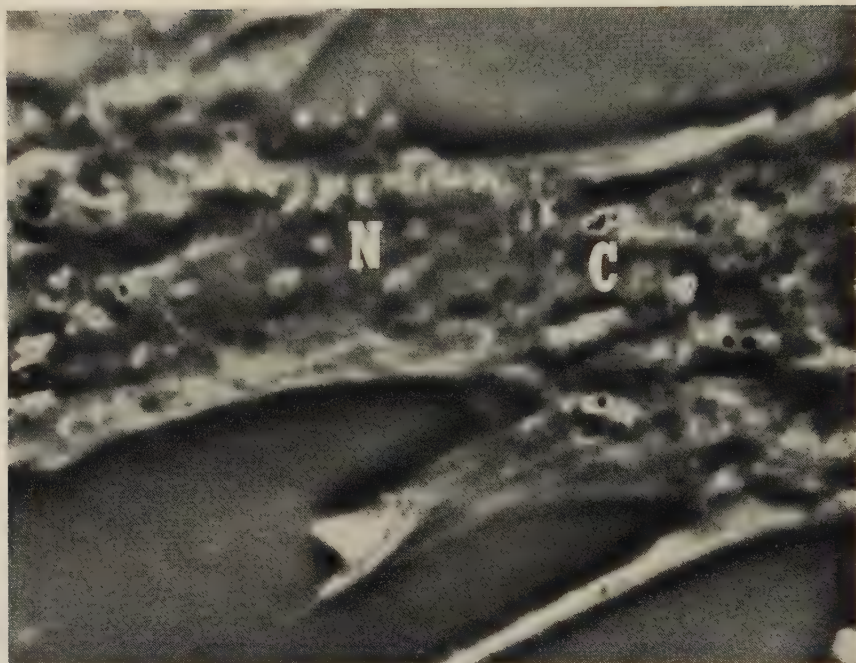


Fig. 6.—Living cells in tissue culture of human placenta, photographed in plastic perfusion chamber mounted on phase contrast microscope. The nuclear membrane and several nucleoli are evident. Enlarged from 16 millimeter motion picture negative. C, Cell body. N, Nucleus.

obtained by thorough washing as is customary for tissue culture glassware, soaking in 95 per cent alcohol for a day, and drying in an oven at 65°C. Ultraviolet sterilization, as suggested elsewhere [7], might be preferred by some.

The ducts are sealed with rubber plugs made for pharmaceutical bottles.¹ This may be done before sterilization, or the plugs may be autoclaved separately and inserted at the time of loading. White rubber has been specified for minimal toxicity. The coverglasses are sealed in place with a beeswax-paraffin mixture flowed on with a hot spatula, and the tissue explant is mounted on the upper window. The plugs are pierced aseptically by two hypodermic needles and medium is introduced. The needles may be withdrawn thereafter, or left in place for perfusion. In either event an effective seal is maintained and there is scant danger of contamination. Bubbles may be diverted from the tissue-bearing area to the ducts by rocking the chamber slightly.

¹ Rubber stopper No. 73-157, in No. 124 white formulation, supplied through the courtesy of The West Company, Phoenixville, Pennsylvania.

The chamber assembly may be clamped with ease on any standard mechanical stage, and the medium manipulated without hindrance to microscopy or photography. The optical path length depends primarily upon the thickness of the core plate, and may be made as thin as 1 mm.

In this laboratory, the perfusion chamber has been used for time-lapse cinematographic studies on a Leitz Ortholux phase contrast microscope, with the entire system enclosed in an insulated box maintained thermostatically at 37°C. Results have been satisfactory, as shown in Fig. 6, enlarged from a representative 16 mm frame.

SUMMARY

A simple and inexpensive laminated plastic perfusion chamber has been developed for tissue culture applications, with emphasis on time-lapse cinematography. Commercial Plexiglas has been found a satisfactory material. Details of fabrication and manipulation are furnished.

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RÔLE DES ACIDES AMINES DANS LA SYNTHÈSE DES ACIDES NUCLÉIQUES CHEZ *ESCHERICHIA COLI*

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TANDIS qu'un grand nombre de travaux ont été consacrés au rôle des acides nucléiques dans la synthèse des protéines [4], le problème concernant la nécessité éventuelle des synthèses protéiniques pour la formation des acides nucléiques n'a été abordé jusqu'ici que de façon préliminaire et a conduit à des résultats quelque peu contradictoires.

En effet, bien qu'on ait réussi à démontrer chez des bactéries en présence de chloromycétine, une synthèse abondante d'acides nucléiques sans formation concomitante de protéines [8, 23], divers auteurs ont mis en évidence une relation obligatoire entre les aminoacides et la synthèse des polynucléotides *in vivo* : Gale par exemple [9] ne constate aucune accumulation d'ARN chez un staphylocoque en milieu synthétique à moins d'ajouter un mélange complet d'acides aminés. Sands et Roberts [21] ont étendu cette observation au cas d'acides aminés spécifiques, puisque selon eux la carence en histidine chez un auxotrophe de *Coli*, ne permet qu'une formation ralentie d'acides nucléiques. Borek [3] enfin, parvient à des résultats similaires avec des auxotrophes différents (à l'exception toutefois d'un mutant particulier d'*E. coli* K. 12 exigeant la méthionine, et accumulant abondamment de l'ARN pendant la carence en acide aminé).

Il existait donc bien un certain paradoxe dans le fait qu'une bactérie peut former ses acides nucléiques sans qu'il y ait synthèse nette de protéines (comme les résultats avec la chloromycétine en font foi), mais requiert cependant pour cette synthèse la présence de tous les acides aminés.

Cette situation conduisait à supposer que les acides aminés participent en tant que tels à la biosynthèse des polynucléotides, c'est-à-dire sans être incorporés en liaison peptidique.

Les observations de Pardee [18] et les nôtres [10] ont permis de démontrer la validité de cette hypothèse, grâce à des études sur les effets combinés de la chloromycétine et d'une carence en acide aminé, dans la synthèse d'acides nucléiques chez divers auxotrophes bactériens.

Nos résultats ont déjà fait l'objet d'une publication préliminaire. Le présent travail apporte une description détaillée de nos expériences, ainsi qu'une série de données nouvelles sur la spécificité d'action des aminoacides.

MATERIEL ET TECHNIQUES UTILISES

Souches utilisées.—Les souches d'auxotrophes utilisées sont les suivantes : *E. coli* M 83 5 (Phénylalanine), *E. coli* ML 304 G (tryptophane), *E. coli* ML 328 D (méthionine), *E. coli* ML 54 (Thrénine), *E. coli* ML 18 (proline), *E. coli* K 678 W 9 (Leucine, Thrénine, B1).

Nous avons eu recours également à une souche de Coli non dépendante d'acides *E. coli* ML 308.

Milieux.—D'une manière générale, les bactéries étaient cultivées en milieu minéral « 56 » sans chlorure de calcium [15], renfermant du maltose (0,2 %) comme source de carbone, ainsi que l'acide aminé exigé en concentration adéquate (Phénylalanine, 10^{-4} M, Tryptophane, 2×10^{-4} M, Méthionine, 5×10^{-5} M, Thrénine, 10^{-4} M, Proline, 10^{-4} M; et dans le cas du mutant à double exigence : Leucine, 10^{-3} M, Thrénine, 10^{-3} M).

Dans les expériences nécessitant un milieu pauvre en phosphates, nous avons utilisé un milieu au « Tris » de composition suivante : Tris (Hydroxyméthyle aminométhane) M/10 pH 7,5, NH_4Cl M/50, KH_2PO_4 M/1000, MgSO_4 M/1000 Fe_2Cl_6 M/100.000.

Préparation des suspensions carencées

Une culture agitée 10 heures (un peu avant la phase stationnaire) est diluée dans du milieu frais à la densité optique de 0,1 à 420 λ . La suspension est incubée à 32°C avec agitation jusqu'à une densité voisine de 0,5. Les bactéries sont alors recueillies par centrifugation à froid, lavées 3 fois en milieu « 56 », et suspendues dans ce milieu minéral renfermant du maltose, mais sans acide aminé. La suspension est distribuée en une série d'aliquotes dans des fioles maintenues dans un bain à 37°C et constamment agitées. La carence est jugée suffisante quand l'accroissement en densité optique devient négligeable, ce qui requiert des durées variables n'excédant généralement pas 30 minutes.

Mesures de la croissance et méthodes de dosage utilisées

La croissance est mesurée par détermination de la densité optique à 420 λ (Beckman). Pour doser les acides nucléiques, une partie aliquote de la suspension bactérienne est additionnée d'acide trichloracétique (concentration finale 5 %). Après 60 minutes à zéro C, on centrifuge, élimine le surnageant, et soumet le résidu à un lavage par l'acide trichloracétique à 5 %. Les acides nucléiques sont extraits en suspendant le résidu lavé, dans de l'acide trichloracétique à 5 %, et en portant l'émulsion 100°C pendant 30 minutes. Le RNA est dosé dans l'extrait selon Albaum et Umbreit [1], le DNA selon Burton [5].

Le résidu final de cette extraction comprend les protéines. Après lavage dans l'acide trichloracétique à 0,2 %, puis dans l'alcool à 60 %, on le dissout dans de l'ammoniaque

dilué et détermine la densité optique à 280 λ . On en déduit la quantité de protéine par étalonnage à l'aide d'une solution dans l'ammoniaque d'ovalbumine cristallisée.

Mesures d'incorporation d'isotopes radioactifs

Nous avons fréquemment mesuré le taux de synthèse des acides nucléiques en déterminant le degré d'incorporation d'Adénine 8- C_{14} dans les bactéries.

Dans certains cas, la radioactivité fut mesurée dans l'extrait des acides nucléiques en milieu trichloroacétique, après avoir dilué et déposé une fraction aliquote sur une coupelle en aluminium.

Le plus souvent, la suspension des bactéries marquées était additionnée de TCA (concentration finale 5 %) et, après 15 minutes, on filtrait sur membranes millipores, puis on lavait plusieurs fois le filtre au TCA à 5 %.

Ces méthodes fournissent d'ailleurs des résultats identiques puisque la quantité de radioactivité provenant de l'adénine et incorporée dans les protéines est une fraction négligeable (1 %) de la quantité totale incorporée dans les bactéries [20].

Pour déterminer le degré d'incorporation d'un aminoacide marqué dans les protéines, la radioactivité était mesurée soit dans la suspension des corps bactériens après lavages répétés à l'eau bouillante, soit dans le résidu protéinique provenant d'une extraction des bactéries par l'acide trichloroacétique chaud et après avoir dissous le résidu dans l'ammoniaque diluée.

RÉSULTATS

Rôle de l'acide aminé essentiel dans la synthèse des acides nucléiques chez des auxotrophes de Coli

L'étude de ce problème a été entreprise avec une assez grande variété de souches présentant soit une exigence unique pour des aminoacides de familles généralement éloignées : Tryptophane, phénylalanine, méthionine, thréonine, proline, soit une double exigence (cas d'un mutant requérant simultanément le thréonine et la leucine). Les organismes prélevés pendant la phase exponentielle de croissance sont carencés selon les conditions décrites, par lavages suivis d'une incubation en milieu minéral renfermant du maltose, mais pas d'acide aminé. On mesure les quantités d'acides nucléiques accumulés dans chacun des cas. Les résultats concernant les mutants simples sont groupés dans le Tableau I.

En présence d'acide aminé, la synthèse d'acides nucléiques est immédiate et généralement exponentielle. En l'absence de l'acide aminé exigé, on n'observe que des accroissements nuls ou négligeables en acides nucléiques. On ne note pas non plus de diminution appréciable dans les limites d'observation (2 à 3 heures).

L'expérience réalisée avec le mutant multiple (Fig. 1) confirme ces conclusions, puisqu'il faut fournir les deux aminoacides pour observer une

TABLEAU I. *Nécessité de l'acide aminé essentiel pour la synthèse d'acides nucléiques chez divers auxotrophes d'Escherichia coli.*

Souche utilisée	Aminoacide exigé	Acide nucléique dosé	Concentration en aminoacide essentiel	Quantité d'acide nucléique (μg pour 10 ml)		% d'accroissement
				0 mn	120 mn	
ML 328 d	Méthionine	ARN	0	238	270	13,4
			$5 \times 10^{-5} M$	—	676	184,0
		ADN	0	22,0	25,7	16,8
			$5 \times 10^{-5} M$	—	76	245,4
ML 36	Méthionine	ARN	0	180	182	1,1
			$5 \times 10^{-5} M$	—	656	264,4
		ADN	0	33,0	37,0	12,1
			$5 \times 10^{-5} M$	—	104,0	215,1
ML 304 G	Tryptophane	ARN	0	332	319	-3,6
			$2,5 \times 10^{-4} M$	—	1092	228,9
		ADN	0	35	42	20
			$2,5 \times 10^{-4} M$	—	95	171
M 83 5	Phénylalanine	ARN	0	590	647	9,6
			$10^{-4} M$	—	1107	87
		ADN	0	72	85	18
			$10^{-4} M$	—	196	172
ML 54	Thréonine	ARN	0	160	184	15
			$2 \times 10^{-4} M$	—	390	143
		ADN	0	—	—	—
			$2 \times 10^{-4} M$	—	—	—
ML 18	Proline	ARN	0	95	127	33
			$2 \times 10^{-4} M$	—	320	236
		ADN	0	—	—	—
			$2 \times 10^{-4} M$	—	—	—

accumulation d'acides nucléiques. La présence d'un seul d'entre eux est insuffisante.

Nous avons recherché si, pendant la carence, on n'observait pas de renouvellement métabolique des acides nucléiques, malgré l'absence de synthèse nette : les expériences décrites dans le Tableau II permettent d'exclure cette éventualité. D'une part, on n'observe pas d'incorporation appréciable de phosphore radioactif ou d'Adénine C_{14} dans les acides nucléiques au cours de la carence. D'autre part, si les acides nucléiques sont préalablement

marqués par un précurseur radioactif pendant la croissance d'un auxotrophe en présence de l'acide aminé essentiel, et que l'on soumette ensuite les organismes à une carence dans un milieu ne renfermant plus ce précurseur, on ne constate aucune perte de radioactivité totale de la fraction des acides nucléiques même après plusieurs heures.

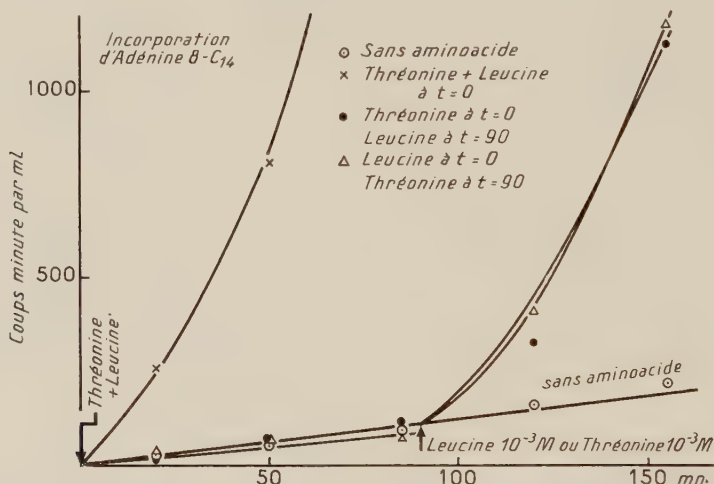


Fig. 1.—Nécessité des deux aminoacides essentiels pour la synthèse d'acides nucléiques chez *E. coli* W 678 79 exigeant la Thréonine et la Leucine (Bactéries carencées 60 minutes avant le début de l'expérience en présence de glucose et de vitamine B1).

Il n'y a donc pendant la carence ni accroissement net, ni renouvellement appréciables des acides nucléiques.

L'ensemble de ces faits prouvent qu'une cellule bactérienne ne peut donc synthétiser d'acides nucléiques que si tous les aminoacides entrant dans la composition de ses protéines sont à sa disposition dans le milieu.

Effets combinés de la chloromycétine et d'une carence en aminoacide essentiel sur la synthèse du RNA

Ainsi que nous l'avons fait remarquer dans l'introduction, la nécessité des aminoacides que nous confirmons ici ne pouvait pas être expliquée par l'existence d'une cosynthèse obligatoire des protéines et des acides nucléiques puisque des expériences indépendantes avec la chloromycétine avaient démontré qu'on peut établir une déconnection pratiquement complète entre la synthèse de ces deux constituants. Les aminoacides devaient donc jouer un rôle indépendamment de leur transformation en protéines.

TABLEAU II. Absence d'incorporation de précurseurs radioactifs dans les acides nucléiques pendant la carence en aminoacide chez un auxotrophe.

Expérience n° 1.—*E. coli* ML 83 5 est cultivé en milieu « Tris » (phosphates 10^{-3} M), renfermant du maltose (2 %) et de la phénylalanine 10^{-4} M. Quand la densité optique atteint 0,85 à 420 m μ , les bactéries sont récoltées à froid, lavées trois fois en milieu Tris, puis suspendues dans le même milieu sans aminoacide à la densité de 0,63. La suspension est divisée en 2 lots l'un demeure sans addition d'acidoacide, l'autre reçoit de la phénylalanine 10^{-4} M. Au temps $t = 0$ les 2 suspensions sont placées à 37°C et reçoivent du phosphate radioactif 25,000 coups min/ml. Incubation pendant des durées variables. Les acides nucléiques sont extraits selon Schneider.

Expérience n° 2.—Mêmes normes que expérience n° 1, mais les bactéries furent cultivées, puis lavées et enfin suspendues en milieu 63 + maltose 2 %. Au temps $t = 0$ on ajoute de l'adénine C¹⁴ (quantité par ml 20 μ g, activité 250 coups min/ μ g).

Echantillons (min.)	Millimicromoles incorporées ds. les acides nucléiques (par ml)	Acides nucléiques	
		Densité optique à 260 de la fraction soluble à chaud ds. le TCA	RNA μ g
Sans phénylalanine	Phosphore radioact.		Méthode à l'orcine
0	—	0,76	41,6
60	21,0	0,65	43,0
120	24,7	0,60	38,8
Avec phénylalanine			
0	—	0,76	41,6
60	185,5	1,92	95,3
120	601,7	3,35	171,0
Sans phénylalanine	Adénine C ₁₄		μ g/ml
0	—	0,700	37,5
60	2,5	0,685	—
120	3,2	0,704	39,5
Avec phénylalanine			
0	—	0,700	37,5
60	26,8	1,400	77,3
120	74,7	2,251	118,3

Tel est le mécanisme que nous essayons d'analyser dans les expériences suivantes.

Restauration de la synthèse des acides nucléiques en présence de chloromycétine chez un auxotrophe carencé en aminoacide. Cinétique de la restauration.—Considérons une suspension d'un auxotrophe carencé en son aminoacide essentiel (tryptophane). Nous savons qu'aucune synthèse d'acide nucléique ne se manifeste dans un tel système. C'est ainsi qu'on n'observe aucun accroissement relatif en composés absorbant à 260 m μ , pas plus d'ailleurs qu'en pro-

téines (absorbant à $420\text{ m}\mu$), contrairement à ce qui se passerait en présence de l'acide aminé exigé (voir Fig. 2).

De la chloromycétine est alors ajoutée dans la suspension carencée en quantités connues comme suffisantes pour inhiber toute synthèse de pro-

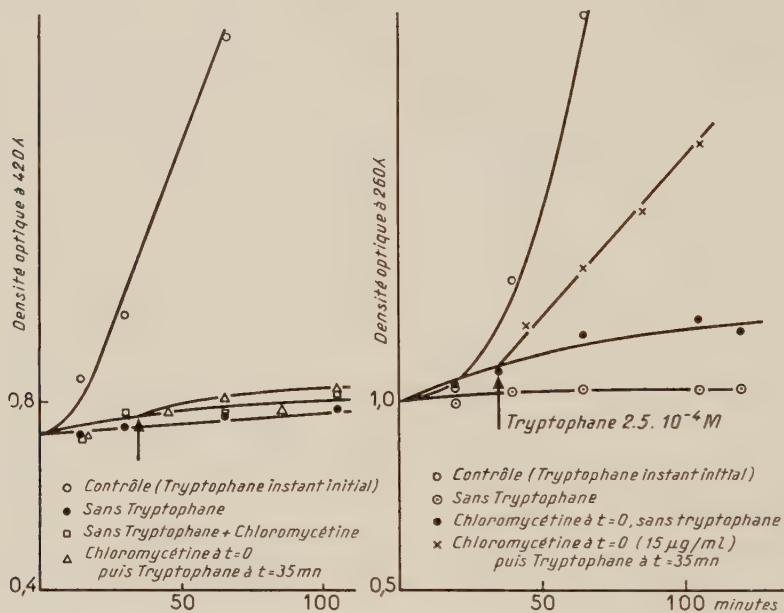


Fig. 2.—Accroissements des densités optiques à 420 Å et 260 Å , chez *E. coli* ML 304 G carencé, après addition de Tryptophane en présence de chloromycétine. Les bactéries sont lavées à l'eau distillés (2°C) et suspendues dans l'eau avant chaque mesure. Les densités optiques à 260 Å sont les valeurs corrigées pour l'effet de dispersion de la lumière à cette longueur d'onde.

téines. Cette addition n'entraîne à elle seule qu'un faible accroissement de densité optique à $260\text{ m}\mu$ et un accroissement négligeable à $420\text{ m}\mu$. Si, par contre, du tryptophane est ajouté quelque temps après l'antibiotique, on provoque immédiatement une accumulation de matériel absorbant à $260\text{ m}\mu$ tandis que la densité à 420 demeure constante (voir Fig. 2). Cette expérience démontre, de façon préliminaire, que l'addition d'acide aminé en présence de chloromycétine restaure une abondante synthèse d'acides nucléiques.

Nous avons cherché à déterminer la nature des acides nucléiques accumulés dans ces conditions par des dosages spécifiques après extraction des polynucléotides dans l'acide trichloroacétique à chaud. De l'ensemble des données réunies dans les Fig. 3, 4 et 5 ainsi que dans le Tableau III, il ressort que seule la synthèse de l'ARN se trouve restaurée, puisqu'aussi bien chez

le mutant exigeant la méthionine, que chez ceux dépendant du tryptophane ou de la phénylalanine, on n'observe aucune restauration de la synthèse du DNA. La synthèse d'ARN qui est rétablie, en présence de chloromycétine,

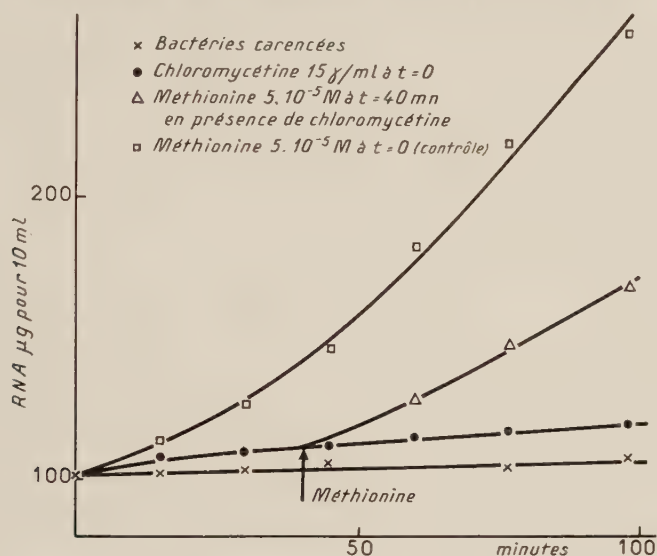


Fig. 3.—Restauration de la synthèse d'ARN après carence, chez *E. coli* ML 328 d, par addition de *dl*-méthionine en présence de chloromycétine. Bactéries carencées en aminoacides pendant 40 minutes avant le début de l'expérience.

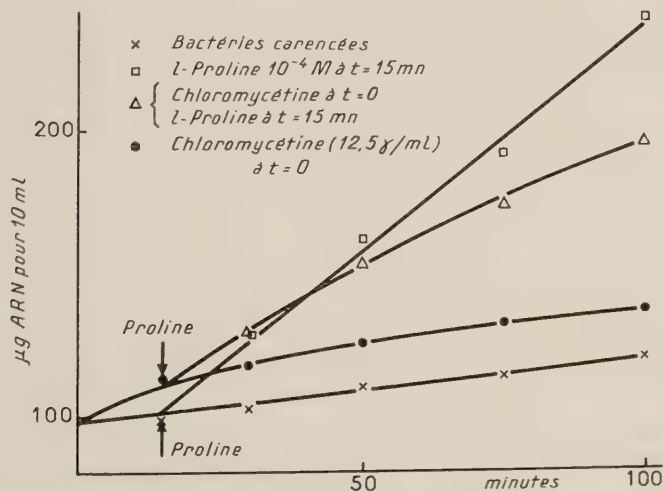


Fig. 4.—Restauration de la synthèse d'ARN après carence, chez *E. coli* ML 1.8, après addition de *L*-proline en présence de chloromycétine.

n'a lieu, il est vrai, que pendant une durée limitée. Son taux initial est en général légèrement supérieur au taux de synthèse normal, mais ce taux diminue ensuite de façon sensiblement exponentielle, la limite atteinte correspondant à un accroissement d'environ 100 % (voir Fig. 5).

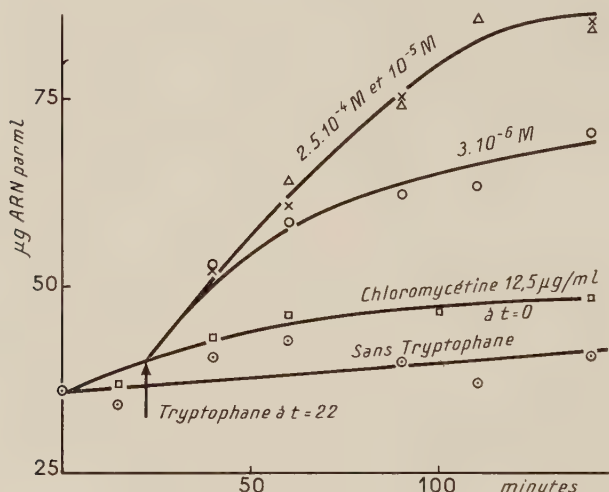


Fig. 5.—Effets de restauration de la synthèse d'ARN chez *E. coli* ML 304 G, pour diverses concentrations de *l*-tryptophane, en présence de chloromycétine.

La cause de cet arrêt est inconnue : d'une part, il ne s'agit pas d'une limitation en aminoacide essentiel qui, dans cette expérience, est ajouté en excès (d'ailleurs l'addition de nouvelles quantités d'acide aminé essentiel après l'arrêt de la synthèse d'ARN ne fait pas repartir celle-ci). D'autre part, le facteur limitant ne réside pas dans l'inactivation d'un système enzymatique par l'antibiotique lui-même. On constate, en effet, que le taux de synthèse de l'ARN, comme son pourcentage d'accroissement final, sont identiques, que l'addition d'acide aminé essentiel ait lieu après un contact de quelques minutes ou de quelques heures entre les bactéries carencées et la chloromycétine. Quoi qu'il en soit, l'ensemble des faits exposés jusqu'ici démontre qu'une synthèse d'ARN peut être rétablie pendant une durée limitée, même si l'addition d'acide aminé se fait en présence de chloromycétine.

Devenir de l'acide aminé essentiel pendant le phénomène de restauration.— Les expériences qui viennent d'être décrites suggèrent que l'acide aminé essentiel peut restaurer la synthèse du RNA sans être incorporé dans les protéines. Il était dès lors important de préciser l'ordre de grandeur des synthèses protéiques, qui subsistent aux concentrations de chloromycétine utilisées. Le taux de ces synthèses a été déterminé chez divers mutants en mesurant le degré d'incorporation d'un acide aminé radioactif dans la fraction

protéinique (voir Tableau IV). Lorsque l'acide aminé essentiel est en excès, ce taux en présence d'antibiotique ne dépasse pas 1 ou 3% de la valeur du contrôle. Il peut atteindre des valeurs élevées si l'acide aminé est en quantités « limitantes ». Ce taux dépend d'ailleurs du mutant considéré.

On peut naturellement se demander si cette synthèse résiduelle ne suffit pas à expliquer l'effet de restauration. Nous allons examiner ce point.

Relations entre l'utilisation de l'acide aminé essentiel pour la synthèse des protéines et son intervention dans la synthèse du RNA.—Supposons qu'une synthèse de protéines même très limitée soit requise pour l'intervention de l'acide aminé essentiel dans la synthèse du RNA. L'addition *en absence de chloromycétine* d'une certaine quantité de cet acide aminé à des bactéries carencées devrait alors provoquer une synthèse d'ARN au moins égale ou supérieure à celle observée quand l'antibiotique est présent. L'expérience montre qu'il n'en est rien, au contraire.

C'est ainsi que l'addition de tryptophane à la concentration de 3×10^{-6} M dans une suspension d'un auxotrophe carencé ne se traduit en absence de chloromycétine que par une synthèse négligeable d'ARN. En présence d'antibiotique, au contraire, la même quantité d'acide aminé ajoutée au même poids de bactéries provoque un accroissement d'au moins 65% en ARN (Fig. 5).

Des expériences en tous points similaires ont été réalisées avec le mutant exigeant la phénylalanine (Fig. 6).

Ces résultats démontrent que les synthèses protéiniques, loin de favoriser l'action de l'acide aminé essentiel, diminuent considérablement ses effets, au contraire, en l'épuisant graduellement du milieu.

Dans ces conditions, on doit admettre ou bien que la synthèse résiduelle des protéines en présence de chloromycétine est étrangère à l'effet de l'acide aminé essentiel sur la synthèse du RNA (et n'aurait tout au plus pour conséquence que de limiter cet effet), ou bien que, contrairement à la majorité des protéines cellulaires, cette fraction résiduelle joue un rôle particulier dans l'élaboration des polynucléotides.

Relation entre le taux de synthèse de l'ARN en présence de chloromycétine et la concentration en acide aminé essentiel.—Que les acides aminés interviennent à l'état « libre » ou par l'intermédiaire d'une protéine spéciale, il était intéressant de déterminer la quantité d'ARN dont ils permettent la synthèse, en présence de chloromycétine. On pouvait espérer préciser, en effet, si leur rôle est catalytique ou s'ils participent de façon stoechiométrique dans la réaction.

Nous avons déterminé dans ce but la quantité totale d'adénine C_{14} incor-

porée dans les acides nucléiques, en présence de chloromycétine, et pour une quantité donnée de phénylalanine, chez l'auxotrophe correspondant.

Des expériences préliminaires ont montré (Fig. 7) que, lorsqu'on met en œuvre de très faibles quantités d'acide aminé (entre 2×10^{-7} et 10^{-6} M),

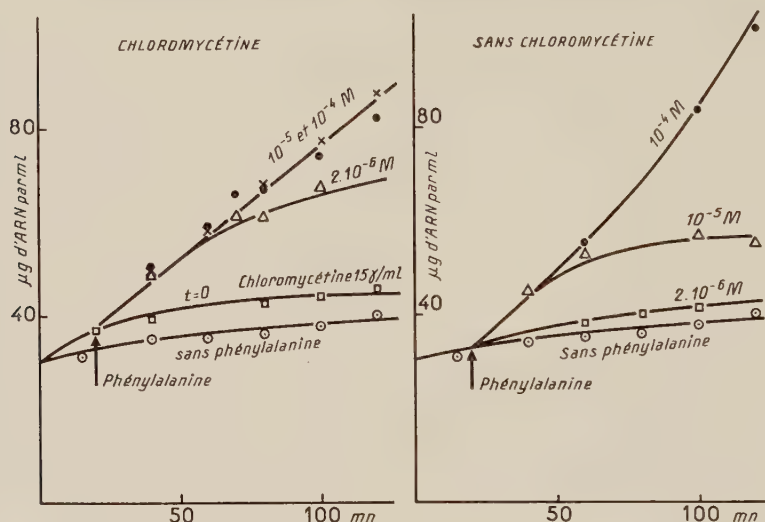


Fig. 6.—Accroissements relatifs en ARN pour de très faibles doses d'acide aminé essentiel en absence ou en présence de chloromycétine, chez *E. coli* M 83 5 (phénylalanine exigeant).

la quantité d'adénine incorporée après un temps donné, ou, si l'on préfère, le pourcentage d'accroissement du RNA, est sensiblement proportionnel à la quantité d'acide aminé ajouté. Cette relation stoechiométrique est due (ainsi que nous l'avons démontré en utilisant le même acide aminé essentiel à l'état radioactif — Phénylalanine C_{14}) à l'épuisement de l'acide aminé lors de la formation des protéines résiduelles. L'addition d'une nouvelle quantité d'acide aminé, une fois la synthèse d'ARN arrêtée, provoque d'ailleurs un accroissement supplémentaire de cet acide nucléique.

Si l'on calcule, à présent, la quantité totale d'adénine incorporée dans l'ARN pour une quantité limitante de phénylalanine, on trouve qu'une molécule de phénylalanine permet l'incorporation de 40 molécules de base. Il s'agit ici de la valeur maxima obtenue parmi diverses expériences; ce chiffre peut varier vraisemblablement avec l'état physiologique des bactéries (degré de carence, etc.).

En admettant une équimolarité des 4 bases dans l'acide nucléique de

E. coli, chaque molécule de base incorporée rend possible l'incorporation de trois autres molécules. On peut donc dire que l'addition d'une molécule de phénylalanine à l'auxotrophe permet l'incorporation de 160 molécules de bases, ou, si l'on préfère, la polymérisation de 160 mononucléotides.

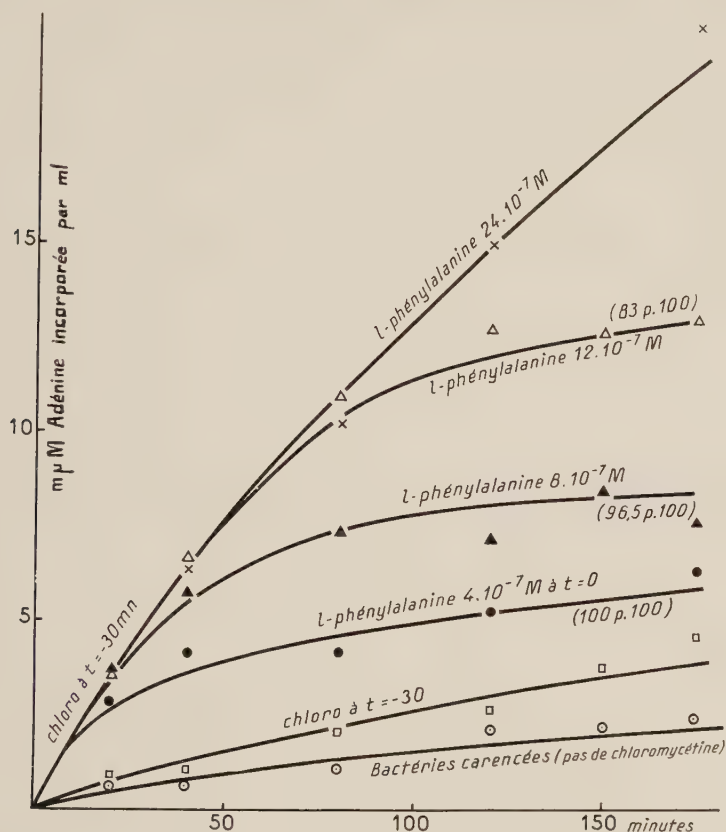


Fig. 7.—Cinétique d'incorporation d'adénine C_{14} dans l'acide ribonucléique en présence de chloromycétine et pour diverses concentrations en phénylalanine (*E. coli* M 83 5). (Les chiffres entre parenthèses représentent les pourcentages d'incorporation de la *l*-phénylalanine dans les protéines après 160 minutes.)

Mais il existe environ 20 aminoacides de types différents dans une cellule, et nous savons que la synthèse des acides nucléiques requiert la présence *simultanée* de chaque aminoacide. On arrive ainsi à la conclusion qu'en moyenne une molécule d'un aminoacide quelconque permet dans les conditions décrites, la polymérisation de 8 nucléotides.

La signification de ce résultat sera discutée ultérieurement.

*Conditions permettant la synthèse du DNA chez un auxotrophe
en présence de chloromycétine*

Comme nous l'avons signalé, l'addition d'acide aminé essentiel dans une suspension carencée renfermant de la chloromycétine ne rétablit pratiquement que la synthèse du RNA et ne provoque aucun accroissement en DNA (Tableau III).

En faisant varier cependant les conditions de notre expérience de restauration, nous avons abouti à des résultats différents.

Au lieu de faire précéder l'addition d'acide aminé essentiel dans la suspension carencée par celle de la chloromycétine, l'acide aminé était ajouté soit en même temps, soit, au contraire, avant l'antibiotique. On mesura dans

TABLEAU III. *Défaut de restauration de la synthèse du DNA en présence d'acide aminé essentiel et de chloromycétine chez un auxotrophe préalablement carencé.*

Les organismes sont prélevés en phase exponentielle (milieu 63 + maltose 2 % et acide aminé exigé), lavés, et carencés dans les conditions décrites page 105. La suspension est alors divisée en plusieurs lots. L'un sert de contrôle de carence, les autres sont additionnés d'acide aminé essentiel, de chloromycétine, ou successivement de ces 2 substances dans les conditions décrites ci-dessous. DNA dosé par la méthode de Burton [5].

Souches auxotrophes d' <i>E. coli</i> utilisées		Additions		DNA μ g pour 10 ml						
		Min.		0	15	40	60	90	110	140
ML 304 G (tryptophane)	Bactéries carencées			47	45	—	54		57	57
	Chloromycétine (15 μ g/ml) à $t=0$				43	43	57		57	57
	Chloromycétine à $t=0$; L-tryptophane 2×10^{-4} M									
	à $t=22$					48	60	54	49	54
	L-tryptophane 2×10^{-4} M à $t=22$					49	60	78	95	107
		Min.		15	40	60	80		100	120
M 83 5 (phénylalanine)	Bactéries carencées			59	62	—	59		—	56
	Chloromycétine (12,5 μ g/ml) à $t=0$				57	57	60		69	68
	Chloromycétine à $t=0$; L-phénylalanine 10^{-4} M à $t=30$				63	58	76		74	74
	L-Phénylalanine 10^{-4} M à $t=30$				70	80	95		119	160

TABLEAU IV. Effet de la chloromycétine sur l'incorporation d'un aminoacide essentiel radioactif dans les protéines d'un auxotrophe carencé.

Dans l'expérience n° 1, l'acidoacide exigé est ajouté à l'état radioactif en concentrations non limitantes pour une croissance normale. On détermine la cinétique de son incorporation dans les protéines, en présence ou en absence de chloromycétine. Dans l'expérience n° 2, avec un auxotrophe différent, nous avons fait figurer les pourcentages d'incorporation de l'acidoacide essentiel pour diverses concentrations de celui-ci, en présence ou en absence de chloromycétine. Pour les mesures de radioactivité, les bactéries sont centrifugées à froid et débarrassées de leur pool cellulaire par deux extractions de 5 minutes dans l'eau bouillante. On mesure la radioactivité dans le résidu après dispersion dans l'eau.

Expérience n° 1.

Souche utilisée	Additions	m μ M de méthionine incorporé par ml (min.)						
		15	30	45	60	75	100	128
<i>E. coli</i> ML 328 D (Méthionine)	Chloromycétine à $t=0$ (15 μ g/ml) Méthionine S ₃₅ à $t=40$	—	—	0,11	0,22	0,27	0,36	0,47
	<i>l</i> -Méthionine S ₃₅ à $t=0$ (15 $\times 10^{-5}$ M) (10 ⁵ coups- minute/micromole)	2,0	4,7	8,0	10,7	16,5	23,9	40,5

Expérience n° 2.

Souche utilisée	Additions	m μ M d'acidoacide essentiel incorporés par ml (min.)					% de la quantité initiale incorporée après 180 min.
		20	70	100	140	180	
<i>E. coli</i> M 83 5 (Phénylalanine)	Sans chloromycétine <i>l</i> -phénylalanine C ₁₄ ^a à $t=0$						
	8,5 $\times 10^{-7}$ M	0,76	0,83	0,90	0,85	0,83	100
	10 ⁻⁵ M	3,9	9,45	9,0	10,6	10,4	100
	Avec chloromycétine (30 γ /ml à $t=-30$) <i>l</i> -phénylalanine C ₁₄ à $t=0$						
	2 $\times 10^{-7}$ M	0,070	0,13	0,12	0,14	0,13	65
	4 $\times 10^{-7}$ M	0,075	0,20	0,21	0,23	0,23	57,5
	8 $\times 10^{-7}$ M	0,10	0,27	0,33	0,35	0,37	46,2
	5 $\times 10^{-6}$ M	0,20	0,38	0,46	0,56	0,65	13,0

^a 1,7 $\times 10^{-5}$ coups-minute par micromole.

TABLEAU V. Influence de l'intervalle séparant les additions d'acide aminé essentiel et de chloromycétine chez un auxotrophe, sur les rendements de synthèse de l'ARN et de l'ADN.

E. coli M 83 5 (phénylalanine exigeant) est soumis à une carence en acide aminé essentiel pendant 100 minutes dans les conditions déjà décrites. On ajoute de la phénylalanine (temps initial), ainsi que de la chloromycétine, soit en même temps que l'acide aminé, soit après des durées diverses de croissance en présence de phénylalanine (concentration en phénylalanine 10^{-4} M, chloromycétine 40 $\mu\text{g/ml}$, température 37°C, densité optique de la suspension 0,6 à 420 m μ). Des prélèvements sont effectués à intervalles réguliers pour doser l'ARN et l'ADN [1, 5].

Intervalles séparant les additions de chloromycétine et d'acide aminé essentiel (min.)	ARN			ADN		
	Teneur en ARN au moment de l'addition de chloromycétine ($\mu\text{g/10 ml}$)	Teneurs en ARN en fin de synthèse ($\mu\text{g/10 ml}$)	Pourcentage d'accroisse- ment	Teneur en ADN au début ($\mu\text{g/10 ml}$)	Teneur en ADN en fin de synthèse ($\mu\text{g/10 ml}$)	Pourcentage d'accroisse- ment
0	318	626	96,1	54	57,2	6,0
10	378	786	107,8	57,6	72	25,0
20	490	960	95,8	63,0	82	30,1
40	612	1296	111,7	78,3	106,2	35,6
60	822	1501	82,6	94,5	131,2	37,1

ces expériences les pourcentages d'accroissement final de l'ADN, ainsi que de l'ARN à titre de contrôle. Les conclusions que l'on peut dégager sont les suivantes (Tableau IV) :

— Le rendement maximum de l'ARN synthétisé en présence de chloromycétine est indépendant du fait que l'acide aminé essentiel soit ajouté en même temps que l'antibiotique ou à des intervalles de temps divers avant ce dernier. Ce rendement correspond toujours très sensiblement à un accroissement relatif de 100 %. Ce résultat, nous le savons déjà, demeure d'ailleurs valable dans le cas où l'addition d'acide aminé essentiel survient même longtemps après l'addition de chloromycétine. Il existe donc une constance remarquable dans le pourcentage d'accroissement de l'ARN en présence de chloromycétine.

— Au contraire, l'ordre d'addition de l'acide aminé essentiel et de la chloromycétine dans une suspension carencée, et l'intervalle de temps séparant ces additions, ont une influence considérable sur le pourcentage d'accroissement de l'ADN (Tableau V).

Celui-ci est nul, comme nous l'avons vu, lorsque la chloromycétine est ajoutée avant l'acide aminé essentiel.

Il est très faible (5 %) lorsque les additions sont simultanées. Par contre, il suffit que l'acide aminé essentiel soit ajouté 10 minutes avant la chloromycétine pour que ce rendement atteigne déjà une valeur notable (22 %). Le pourcentage d'accroissement final en ADN augmente un peu avec l'intervalle séparant l'addition d'acide aminé de celle ultérieure de chloromycétine. Dans l'expérience décrite au Tableau V, il atteint un maximum (soit 37 %) lorsque l'acide aminé a été introduit 50 minutes avant l'antibiotique dans la suspension carencée. (De toute manière, l'accroissement moyen maximum ne dépasse pas 50 %.)

En résumé, on n'observe de synthèse d'ADN en présence de chloromycétine que dans un système déjà en train de synthétiser activement certains constituants. La nature de ceux-ci reste à déterminer (protéines ou ribonucléoprotéines). Tout se passe comme si la synthèse d'une faible quantité de protéines (peut-être détruites pendant la carence) était nécessaire au déclenchement de la synthèse d'ADN, celle-ci pouvant se poursuivre dès lors comme la synthèse du RNA, avec la seule présence des acides aminés libres et sans formation concomitante de protéines.

Synthèse de précurseurs d'acides nucléiques chez des auxotrophes en absence de leur acide aminé essentiel

Des expériences ont été réalisées avec le mutant exigeant la phénylalanine en vue de déceler une accumulation possible de précurseurs d'acides nucléiques pendant la carence en acide aminé essentiel.

Tandis qu'en absence d'acide aminé, l'accroissement relatif des acides nucléiques après 120 minutes n'excède pas 2 % et qu'on n'observe pas d'incorporation notable d'adénine C_{14} dans la fraction des acides nucléiques, la quantité de matériel absorbant à 260 $m\mu$ que l'on peut extraire des cellules par l'acide perchlorique à froid, ainsi que le ribose contenu dans cette fraction, s'accroissent d'environ 100 %. L'accroissement relatif de teneur en précurseurs nucléiques (absorbant à 260 $m\mu$) est encore plus prononcé si l'on considère le surnageant après centrifugation des cellules carencées.

Nous avons procédé à l'identification et au dosage des mononucléotides parmi les produits accumulés, à l'aide de fractionnements plus spécifiques.

Les résultats démontrent sans ambiguïté une accumulation considérable de mononucléotides des 4 types dans le pool cellulaire, pendant la carence en acide aminé. Cette accumulation atteint environ 100 % en 120 minutes pour les acides adénylique et guanylique (Tableau VI).

TABLEAU VI. *Accumulation de mononucléotides puriques ou cours de la carence en aminoacide chez un auxotrophe.*

1 litre de culture exponentielle de *E. coli* M 83 5 (milieu « 63 », au maltose + phénylalanine 10^{-4} M à la densité optique de 0,75 (420 λ)) est centrifugé à froid. Les bactéries sont lavées 3 fois en milieu 63 et suspendues à nouveau dans le même milieu, sans aminoacide. On divise en 3 lots de 300 ml chacun. Le premier « A » sert de contrôle pour la composition initiale en nucléotides, le second « B » est soumis à une carence de 2 heures à 37°C, le troisième « C » est additionné de phénylalanine 10^{-4} M et placé à 37°C pendant 2 heures. Les bactéries provenant de A, B et C sont centrifugées à 0°C, lavées une fois au milieu 63 glacé et soumises à deux extractions successives dans 20 ml d'acide perchlorique. Les nucléotides sont séparés des autres composés présents dans l'extrait sous forme de sels d'urane insolubles, puis soumis à une hydrolyse dans SO_3H_2 N/1 pendant 1 heure qui libère les purines. Celles-ci après précipitation sous forme de sels d'argent, sont séparées par chromatographie sur papier (24), puis élues quantitativement par HCl N/1. On dose leur teneur par spectrophotométrie en U.V.

Densité optique de la suspension à 420 λ	Acides nucléiques OD à ^a 260 λ		Accumulation de nucléotides puriques dans le pool cellulaire			
			Acide adénylique		Acide guanylique	
			μg p. 300 ml d'adénine	par unité de masse bactér.	μg de guanine p. 300 ml	corrigés pour l'accrois- sement d'O.D.
Début de la carence, A						
0,639	1,40	37,5	43,2	43,2	61,2	61,2
Après 2 h. de carence en phénylalanine, B						
0,645	1,48	39,5	90,0	90,0	136,2	136,2
Après 2 h. de croissance en présence de phénylalanine, C						
2,235	5,53	118,3	132,0	72,0	102,3	56,8

^a 2 ml de suspension sont précipités par l'acide trichloracétique à 5 % à 0°C. Après un lavage, les acides nucléiques du résidu sont extraits dans 1 ml de TCA à 5 % pendant 30 min. à 100°C.

^b Méthode de Albaum et Umbreit.

Ces composés ne peuvent résulter d'une décomposition d'acides nucléiques, dont la teneur demeure constante au cours de la carence, ainsi que nous l'avons établi par des mesures diverses.

C'est donc bien dans la synthèse d'acides nucléiques à partir de leurs précurseurs, et non dans la synthèse de ces derniers, qu'interviennent les aminoacides.

*Effet du remplacement d'un aminoacide par un analogue
sur le taux de synthèse des acides nucléiques*

Nous venons de voir que les aminoacides jouent un rôle spécifique dans la synthèse des acides nucléiques indépendamment, semble-t-il, de leur transformation en protéines. On savait d'autre part, d'après les observations indépendantes de Munier et Cohen [16], ainsi que de Pardee [18], que certains analogues peuvent être utilisés directement dans la synthèse des protéines à la place des aminoacides naturels apparentés.

On pouvait donc se demander si ce remplacement d'un aminoacide naturel par un analogue aurait les mêmes répercussions sur la synthèse des acides nucléiques que sur celle des protéines.

Ce problème a été étudié soit avec des auxotrophes bactériens en fournissant un analogue à la place de l'acide aminé essentiel, soit chez un prototrophe (*E. coli* ML 308) en ajoutant un excès de l'analogue dans une culture en phase exponentielle. Les résultats relatifs à la parafluorophénylalanine, à l'éthionine ou à la norleucine sont décrits dans le Tableau X, ainsi que dans les Fig. 8 et 9. Ils sont d'ailleurs sensiblement les mêmes, que l'on considère des organismes auxotrophes ou prototrophes.

Une conclusion inattendue s'en dégage : le remplacement de l'acide aminé naturel par l'analogue affecte beaucoup plus les synthèses d'acides nucléiques que celles des protéines.

En effet, le taux de synthèse absolue des protéines est très peu affecté en présence de l'analogue, la seule différence étant qu'au lieu d'être exponentielle, la synthèse des protéines est linéaire en fonction du temps (Tableau VII) dès l'addition d'analogue. Au contraire, les taux de synthèse absolus du RNA et du DNA sont considérablement diminués.

Si donc l'on considère (Fig. 8 et 9) le taux de synthèse des acides nucléiques rapporté à l'accroissement en protéines, ou taux de synthèse différentiel, on constate qu'il est abaissé dans des proportions considérables en présence des analogues. Par exemple, l'inhibition atteint 80 % pour le RNA chez l'auxotrophe quand l'éthionine remplace la méthionine, elle atteint 90 % pour la synthèse du DNA. Ces inhibitions sont un peu moins prononcées dans le cas du prototrophe.

Le remplacement de la phénylalanine par la parafluorophénylalanine, chez un auxotrophe ou un prototrophe, se traduit par des inhibitions du même ordre dans les taux de synthèse différentiels des acides nucléiques.

Ces résultats démontrent donc que les besoins en aminoacides d'une bactérie sont beaucoup plus spécifiques en ce qui concerne la synthèse de RNA

que pour la synthèse des protéines. Nous avons déjà décrit une série de faits qui suggèrent une participation des aminoacides à la synthèse du RNA, indépendamment de leur incorporabilité dans les protéines. On pouvait donc penser, par exemple, que les analogues stériques des aminoacides, sont des agents « activateurs » beaucoup moins efficaces que les aminoacides naturels dans la synthèse des acides nucléiques.

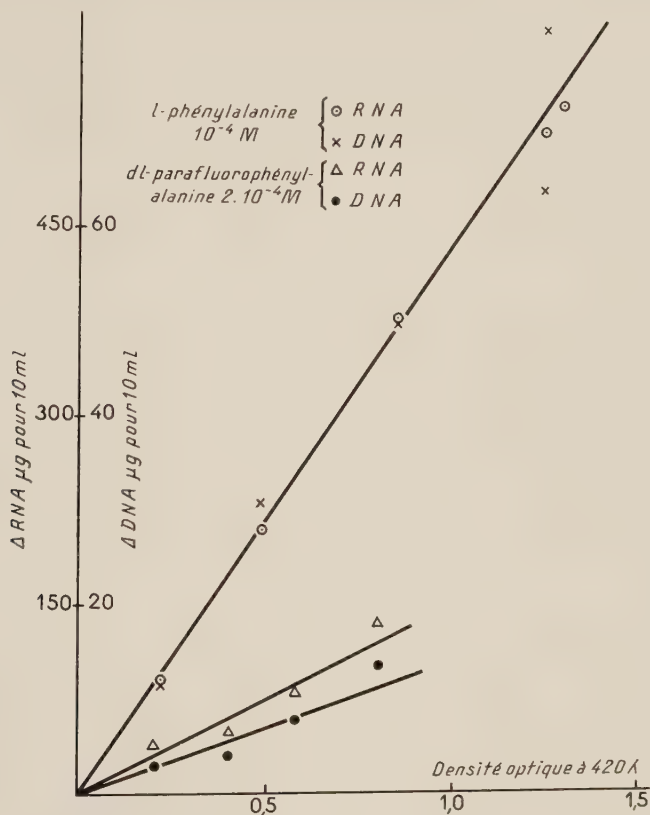


Fig. 8.—Effet du remplacement de la phénylalanine par la parafluorophénylalanine sur les taux différentiels de synthèse des acides nucléiques chez *E. coli* M 83 5.

Pour envisager cette possibilité, nous avons comparé les effets de restauration en présence de chloromycétine, de certains aminoacides essentiels et de quelques analogues correspondants, en répétant les expériences déjà décrites à la page 121.

Effet des analogues en présence de chlormycétine. Spécificité d'action des aminoacides.—L'ensemble des données se trouvent groupées dans le Tableau VIII. L'addition à des auxotrophes carencés de 5-méthyltryptophane, ou d'éthionine en présence de chloromycétine, se traduit par des accroissements

en RNA voisins (quoique un peu inférieurs) de ceux observés après addition des aminoacides naturels. La parafluorophénylalanine, et la phénylalanine manifestent d'ailleurs quantitativement les mêmes effets. Ainsi l'abaissement du taux différentiel de synthèse des acides nucléiques que nous avons observé pendant la synthèse linéaire des protéines (Fig. 8 et 9) en présence d'éthionine ou de parafluorophénylalanine, n'est donc pas dû au manque de

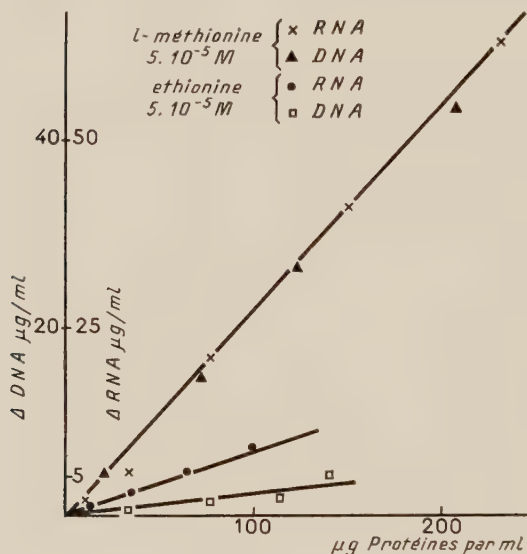


Fig. 9.—Effet du remplacement de la méthionine par l'éthionine sur les taux différentiels de synthèse des acides nucléiques chez *E. coli* ML 328 d.

la spécificité requise pour « activer » la synthèse des acides nucléiques. La cause de cette diminution demeure donc encore inexpliquée. Il existe néanmoins comme dans le cas des aminoacides naturels (p. 113) une compétition entre l'utilisation des analogues « incorporables » pour la synthèse des protéines, et leur intervention spécifique dans la synthèse du RNA. Par exemple l'addition d'une quantité de parafluorophénylalanine trop faible (2×10^{-6} M) pour provoquer, en l'absence d'antibiotique, un accroissement notable des protéines ou des acides nucléiques, permet au contraire, en présence de chloromycétine une abondante synthèse de RNA, chez l'auxotrophe carencé en phénylalanine (accroissement de 50 %).

Ces recherches sur les analogues nous ont amenés à étendre notre étude sur la spécificité chimique des aminoacides comme agents d'activation dans la synthèse d'ARN. Seule notre étude avec le mutant phénylalanine présente jusqu'ici quelque caractère systématique : contrairement à la parafluorophénylalanine qui peut remplacer la phénylalanine, la parahydroxyphényl-

TABLEAU VIII. Effets comparatifs des aminoacides et de certains analogues structuraux dans la synthèse d'ARN en présence de chloromycétine.

Dans les expériences 1 et 2, M 83 5 est cultivé en milieu minéral (+ maltose et phénylalanine). Quand la densité optique est 0,7 (à 420 m μ), les bactéries sont récoltées, lavées 3 fois en milieu minéral, et suspendues dans le même milieu renfermant du maltose mais sans aminoacide. Après 45 min. de carence on ajoute de la chloromycétine, puis 30 min. après addition d'antibiotique, la suspension reçoit l'acide aminé essentiel ou un analogue correspondant, en même temps que de l'adénine 8-C₁₄ (10⁻⁴ M, 225 c.m./ μ g). Dans les expériences 3 et 4, avec des auxotrophes différents, le protocole est à peu près identique, mais l'on détermine directement l'accroissement en ARN.

Souches utilisées	Additions	Incorporation d'adénine 8-C ₁₄ dans les acides nucléiques (m μ M/ml) (min.)		
		30	60	120
M 83-5 (phénylalanine)	Bactéries carencées	1,0	1,8	2,5
	Chloromycétine 12/ml au temps $t = -30$ mn	1,8	2,9	4,5
	Chloromycétine à $t = -30$ mn; <i>l</i> -phénylalanine 5×10^{-5} M à $t = 0$	7,7	12,4	24,8
	Chloromycétine à $t = -30$ mn; <i>dl</i> -para-fluorophénylalanine 10^{-4} M, $t = 0$	8,2	12,4	20,8
M 83 5 (phénylalanine)	Chloromycétine au temps $t = -30$ mn		3,8	6,4
	Chloromycétine à $t = -30$ mn; <i>l</i> -phénylalanine 2×10^{-7} M à $t = 0$		11,0	12,0
	Chloromycétine à $t = -30$ mn; <i>dl</i> -para-fluorophénylalanine 4×10^{-7} M, $t = 0$		14,5	14,7
		RNA μ g/par ml, min.		
		0	120	% d'accroiss.
ML 304 G (tryptophane)	Bactéries carencées	26,2	26,2	0
	Chloromycétine (12 /ml) $t = 0$		29,2	11
	Chloromycétine à $t = 0$; <i>l</i> -tryptophane 10^{-5} M à $t = 20$ mn		41,0	57
	Chloromycétine à $t = 0$; 5-méthyle-tryptophane 10^{-4} M; $t = 20$		36	34
ML 328 d (méthionine)	Bactéries carencées	32,0	34,6	8
	Chloromycétine à $t = 0$		40,0	25
	Chloromycétine à $t = 0$; <i>l</i> -méthionine 5×10^{-5} M $t = 20$		55	71
	Chloromycétine à $t = 0$; <i>l</i> -éthionine 5×10^{-5} M à $t = 20$		48	50

alanine et la parachlorophénylalanine sont inactives. Il en va de même de la thiénylalanine.

Sont inactifs également : la dioxyphénylalanine, la diphénylalanine (et ses dérivés N substitués) ainsi que l' α -phénylalanine et le tryptophane.

La d-phénylalanine est active au contraire à certaines concentrations, mais ce fait est dû à son isomérisation en forme *l*, ainsi que nous avons pu le démontrer dans des expériences indépendantes.

L'étude de l'importance des fonctions carboxylées ou α -aminées de l'acide aminé n'a été, elle aussi, abordée que récemment. La glycyl-phénylalanine est active d'emblée, même à 5×10^{-7} M, tandis que son dérivé la glycyl-phénylalanine-amide s'avère inactif. Le fait que l'ester éthylique de la phénylalanine¹ soit également dépourvu d'activité tendrait donc à suggérer que le blocage du groupe carboxyle fait disparaître l'activité, tandis qu'une substitution sur le groupe α -aminé pourrait la respecter. Ce point demande néanmoins confirmation par des études plus approfondies.

DISCUSSION

Divers auteurs ont mis en évidence une relation générale entre les aminoacides et la capacité de synthétiser des acides nucléiques : c'est ainsi qu'une bactérie requiert, pour élaborer ses polynucléotides, l'ensemble des aminoacides constitutifs de ses protéines, et s'en avère incapable si un seul d'entre eux fait défaut.

Bien que la nécessité des aminoacides ait été ainsi reconnue depuis déjà quelques années, il ne semble pas que l'importance de ce fait ait été perçue tout d'abord, sinon comme la preuve d'une certaine corrélation générale, mais non spécifique, entre la synthèse des protéines et celle des acides nucléiques.

Ce n'est que récemment, depuis les travaux de Pardee [17] et les nôtres [10] que l'on s'est trouvé amené à analyser le phénomène en détail et à réviser totalement l'interprétation précédente.

Les observations qui ont abouti à ce résultat peuvent être résumées de la manière suivante : chez un auxotrophe carencé en aminoacide et, par conséquent, incapable d'accumuler des polynucléotides, on peut restaurer une synthèse d'acide nucléique en ajoutant l'acide aminé exigé, même en présence de chloromycétine à doses suffisantes pour inhiber l'incorporation de cet aminoacide dans les protéines.

¹ Ces 3 composés ont été synthétisés et nous ont été généreusement procurés par le docteur E. Bricas.

Notre travail a permis de préciser, en outre, que, seule, la synthèse de l'ARN était restaurée dans ces conditions. Il s'agit d'une synthèse limitée à un doublement de la teneur initiale en acide nucléique. L'effet de restauration est obtenu avec des quantités extrêmement faibles d'acide aminé essentiel. On peut calculer que chaque molécule d'un acide aminé permet en moyenne l'incorporation de 8 nucléotides dans les acides nucléiques.

Il apparaît donc que les acides aminés ont un rôle très spécifique dans la synthèse du RNA, rôle qui, selon toute vraisemblance, est indépendant de leur incorporabilité dans une chaîne peptidique, puisqu'ils l'exercent en présence de chloromycétine.

Toutefois le blocage des synthèses protéiques par la chloromycétine, s'il est considérable, n'est cependant pas absolu. Il subsiste 2 à 5 % des synthèses normales : l'on pouvait donc se demander si cette synthèse résiduelle de protéine n'était pas suffisante pour permettre une accumulation du RNA. S'il en était ainsi, une certaine quantité limitante d'acide aminé devrait alors provoquer une synthèse d'ARN égale ou supérieure, en l'absence de chloromycétine, à celle observée quand l'inhibiteur est présent. Or, nous l'avons vu, pour de très faibles quantités de l'acide aminé essentiel, la quantité totale d'ARN accumulée dépasse de 10 à 20 fois, en présence de chloromycétine, celle élaborée dans les conditions normales.

Si la fraction des protéines résiduelles intervient donc tant soit peu dans la synthèse du RNA en présence de chloromycétine, il ne peut s'agir que d'une fraction très particulière ayant un rôle spécifique dans la synthèse de cet acide nucléique.

Bien qu'on ne puisse rejeter cette éventualité, certaines données la rendent quelque peu improbable. Tout d'abord, il faudrait pouvoir expliquer pourquoi cette protéine particulière est la seule à pouvoir s'accumuler en présence de chloromycétine. En outre, rien ne permet d'affirmer que les protéines formées en présence de chloromycétine appartiennent à une catégorie particulière. L'ultracentrifugation d'un extrait de bactéries, ayant incorporé un acide aminé radioactif en présence de chloromycétine, montre que les protéines élaborées dans ces conditions se répartissent selon un gradient identique à celui de protéines formées dans des conditions normales. De plus, le degré d'association, avec les acides nucléiques, des protéines accumulées en présence d'antibiotique est pratiquement identique à celui de protéines normales. C'est ainsi que 60 % de ces protéines se retrouvent, après extraction, sous forme de nucléoprotéines aisément centrifugeables et leur liaison avec l'acide nucléique résiste à une action prolongée des ultrasons [11].

Nous considérons donc comme plus plausible l'éventualité selon laquelle les aminoacides exercent un rôle spécifique en tant que tels, c'est-à-dire sous forme libre ou du moins sans être préalablement assemblés en liaisons peptidiques.

Le chiffre trouvé de huit nucléotides polymérisés par molécule d'acide-aminoacide présent semble en faveur d'un mécanisme d'action purement catalytique des aminoacides : chaque aminoacide activerait spécifiquement l'incorporation de mono- ou dinucléotides spécifiques et cet effet ne serait interrompu que par une éventuelle intégration de l'acide-aminoacide dans une protéine.

Que leur rôle soit d'ailleurs stoechiométrique ou catalytique, on est conduit à supposer que les aminoacides forment à un moment donné une combinaison spécifique avec des mononucléotides, puisque chaque aminoacide est nécessaire *individuellement*.

Le mécanisme assurant la formation du complexe hypothétique « aminoacide-nucléotide » est peut-être voisin de celui qui permet l'activation des aminoacides, tel que l'on décrit Hoagland, Berg, de Moss, Novelli, et Lipman [13, 2, 7, 6]. On sait que la forme dite « active » des aminoacides est un anhydride mixte d'acide adénylique et d'acide-aminoacide. On peut donc supposer qu'il se formerait des anhydrides du même type avec d'autres nucléotides et que le clivage de ces composés s'effectuerait de manière à libérer les nucléotides (au lieu des aminoacides) sous une forme activée permettant leur polymérisation ultérieure.

L'existence de complexes « nucléotide-aminoacides » paraît d'ailleurs avoir été démontrée dans certains cas [19]. Il est difficile actuellement de préciser si c'est le groupe carboxyle plutôt que le groupe aminé des aminoacides qui se trouve impliqué dans l'activation des nucléotides. Des études sont actuellement en cours sur ce point. Il semble acquis que la parafluorophénylalanine peut remplacer la phénylalanine, le 5-méthyltryptophane remplacer le tryptophane, et l'éthionine la méthionine. Or, aucun de ces analogues ne peut servir de substrat aux enzymes activant les aminoacides correspondants [7, 22]. Sur ce point, on décèle donc une différence entre le mécanisme d'activation d'un aminoacide et le mécanisme supposé de l'activation des nucléotides par les mêmes aminoacides.

En ce qui concerne la polymérisation des nucléotides après leur combinaison avec les aminoacides, il apparaît comme très vraisemblable que, pour pouvoir exercer spécifiquement un rôle dans la synthèse de l'ARN, chaque aminoacide, avant ou après sa combinaison spécifique avec les nucléotides, doit se trouver maintenu, même provisoirement, à la surface d'une matrice spécifique (protéine ou acide nucléique).

Une fois les nucléotides rattachés à cette matrice rigide par l'intermédiaire des aminoacides, ils se trouveraient réunis par des liaisons phosphoriques et il y aurait formation d'un acide nucléique. La séquence des nucléotides étant complémentaire de celle des aminoacides, l'absence dans le milieu d'un aminoacide de type donné se traduirait par une série de discontinuités dans l'assemblage des nucléotides, qui rendrait impossible leur polymérisation.

Ce modèle permet d'expliquer la nécessité de chaque aminoacide *individuellement et indépendamment de la formation d'une protéine*, puisque les aminoacides sont supposés être fixés transitoirement sur une matrice et non réunis en liaisons peptidiques.

On peut donc admettre que d'une manière générale, les aminoacides sont impliqués dans deux types de réaction; ou bien ils catalysent l'incorporation de nucléotides dans les acides nucléiques selon le mécanisme que nous venons de décrire, ou bien, lorsque les conditions le permettent, ils sont incorporés eux-mêmes en liaisons peptidiques.

Il y a d'ailleurs des raisons de penser que ces deux processus évoluent de façon pratiquement indépendante; sinon, on aboutirait à une co-synthèse stricte des acides nucléiques et des protéines. Or, le fait qu'une cellule renferme de 3 à 4 fois plus de protéines que d'acides nucléiques prouve bien qu'il n'y a pas équivalence entre le nombre de liaisons peptidiques et le nombre de liaisons internucléotidiques.

Il faut toutefois souligner que la synthèse de RNA en présence de chloromycétine est limitée à un accroissement relatif de 100%. L'absence de corrélation entre synthèse de RNA et synthèse de protéines (que nous avons amenés à supposer) n'est donc pas absolue et certains stades au moins de la biosynthèse de l'acide nucléique dépendent peut-être de la formation de certaines protéines spécifiques.

Il en irait de même pour la synthèse du DNA : nous avons en effet signalé dans le présent travail que la synthèse du DNA en présence de chloromycétine est non seulement limitée à un certain accroissement relatif, mais qu'elle dépend, de plus, de la période de croissance au cours de laquelle l'antibiotique est ajouté. La formation de certains constituants protéiniques paraît donc une condition préliminaire à l'accumulation de cet acide nucléique.

En dehors des spéculations que permet ce travail sur le mode de synthèse des acides nucléiques, il révèle également toute la complexité biochimique de ce processus.

A la suite des observations de Grunberg Manago et Ochoa d'une part [12]

de Kornberg, d'autre part [14], on pouvait se représenter la synthèse des acides nucléiques *in vivo* comme une simple polymérisation de nucléotides analogue à celle qu'ont observée ces auteurs *in vitro*. Un tel mécanisme devait, il est vrai, avoir la spécificité requise pour produire des acides nucléiques biologiquement actifs, mais cette objection paraissait pouvoir être écartée en supposant un agencement préalable des nucléotides sur une matrice porteuse de la spécificité.

Les résultats actuels sur la nécessité d'acides aminés spécifiques obligent à conclure que les « polymérases » du type étudié par Grunberg Manago et Ochoa, ou bien ne sont pas fonctionnelles *in vivo*, ou bien requièrent une certaine concentration locale de précurseurs, telle que le permettrait, par exemple, l'assemblage sur une matrice de complexes « acides aminés-nucléotides ».

RÉSUMÉ

La carence en acide aminé essentiel chez divers auxotrophes d'*Escherichia coli* provoque l'arrêt des synthèses d'acides nucléiques. La synthèse de l'ARN peut être restaurée si l'acide aminé essentiel est à nouveau ajouté, même en présence de chloromycétine qui abolit l'incorporation des acides aminés dans les protéines. Le calcul du rendement de synthèses de l'ARN en fonction de la quantité d'acide aminé ajouté suggère un rôle catalytique de ce dernier.

Les acides aminés spécifiques peuvent être remplacés dans ce rôle particulier par des analogues structuraux.

SUMMARY

Amino acid auxotrophs of *E. coli*, after starvation for their growth factor, do not synthesize nucleic acid. The RNA synthesis can be restored if the essential amino acid is added again, even in the presence of chloromycetin. Since this inhibition totally abolishes amino acid incorporation into proteins, this fact suggests that free amino acid plays a role in nucleic acid synthesis. The calculation of the RNA yield synthesized as a function of the amount of amino acid added suggests that this is a catalytic role. Certain amino acid analogues can be substituted for the essential amino acids in this restoration effect.

Nous tenons à remercier bien vivement le docteur Jacques Monod pour les critiques et les suggestions qu'il nous a apportées tout au long de ce travail.

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THYROXINE-MEDIATED RELEASE OF DIPHOSPHOPYRIDINE NUCLEOTIDE FROM MITOCHONDRIAL DEHYDROGENASES

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IN spite of the large amount of work which has been done, the exact nature of the mode of action of thyroxine is still a matter of uncertainty. Martius [19, 20] and Lardy [13, 14] consider the possibility that thyroxine acts as a specific regulator of the "high-energy" phosphate output during biological oxidations. However, following the extensive studies of Lehninger and his associates [15, 26, 27], doubt has arisen whether thyroxine acts on oxidative phosphorylation in any specific way. The latter investigators adduced strong evidence that thyroxine exerts its prime effect on the mitochondrial membrane causing the swelling of the particles. This effect can be demonstrated at a physiological concentration of the hormone [16] in contrast to the uncoupling phenomenon, which is brought about at rather high aphysiological concentrations.

During recent investigations it was found that thyroxine, diethylstilboestrol, and certain hepatocarcinogens inhibited the glutamate oxidation of mouse liver mitochondria, whereas no inhibition was noted in the presence of DPN.¹ The effects of the hepatocarcinogens have already been briefly described [5, 6]. The present communication deals mainly with the experiments carried out with thyroxine under conditions in which the oxidative phosphorylation of the mitochondria was not impaired. The results lend further support to the view that thyroxine acts on the biochemical integrity of the mitochondria in general rather than on the oxidative phosphorylation in particular.

EXPERIMENTAL

Mitochondria were isolated at 0°C according to Schneider and Hogeboom [23] in 0.25 M sucrose from the livers of male and female F_2 (B.CBA $\times$ B.CBA) mice,

¹ Abbreviations are used as follows: DPN, diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate; PVP, polyvinylpyrrolidone.

and washed twice; B.CBA stands for F_1 (♀ C57 black $\times$ ♂ CBA). Incubation was carried out with mitochondria from 200–250 mg fresh weight of liver. The respirometers contained (unless otherwise stated): histidine 0.05 M , MgCl_2 0.005 M , phosphate 0.013 M , KCl 0.038 M , cytochrome c 0.00005 M , AMP 0.0062 M . L-Glutamate (0.035 M) was added from the side-arm after temperature-equilibration had been reached (8 min.). DL-Thyroxine was present in a final concentration of 10^{-6} M to 7.5×10^{-5} M and 10^{-3} M DPN was added as indicated in the text.

Final fluid volume 1.6 ml; pH 7.4. Incubation at 27°C during 20 min. with shaking in air. Oxygen uptake by standard Warburg technique. Inorganic phosphate was determined by the method of Fiske-Subbarow. Glucose, hexokinase and malonate were not present; P/O ratios of around 2 were obtained in all experiments. The oxygen uptakes listed in the tables, which illustrate typical experiments, were corrected for the appropriate blanks.

The medium, minus thyroxine and DPN, is called the standard medium. Variations were made by adding EDTA (10^{-3} or 10^{-2} M), PVP (2 or 7.5 per cent), changing the Mg^{++} concentration to 0.0025 M (designated in the text as the low Mg^{++} medium) or to 0.0075 M and adding MnSO_4 (0.0025 M) to the low Mg^{++} medium. Diethylstilboestrol was dissolved in ethanol and 0.1 ml of the solution was added to the flasks resulting in a final concentration of 5 or 2.5×10^{-5} M of the estrogen. Polyvinylpyrrolidone was a gift from Rhône-Poulenc (France).

RESULTS

The inhibition of glutamate oxidation of mouse liver mitochondria by thyroxine.—Thyroxine inhibited the glutamate oxidation of the liver mitochondria to various degrees. Except for a few experiments, 7.5×10^{-5} M thyroxine effected complete inhibition in mitochondrial preparations containing from 0.80 to 1.30 mg N . 5×10^{-5} M Thyroxine produced complete inhibition in about 60 per cent of the experiments. DPN, routinely added in a final concentration of 10^{-3} M , always reversed the inhibitions. The difference in response of the mitochondrial preparations to thyroxine might have been due to the fact that the livers of F_2 hybrids, which are genetically impure, were used. The data showed that the inhibition produced by thyroxine was inversely related to the mitochondrial integrity involving DPN. The greatest inhibition was always found with those preparations whose glutamate oxidation in the absence of thyroxine was stimulated by the addition of DPN, as illustrated in exp. 1 and 2 of Table I. Exp. 3 of Table I shows the effect of various concentrations of thyroxine on the glutamate oxidation of a rather susceptible mitochondrial preparation. The hormone still showed some inhibitory effect at 10^{-5} M but was inactive at 10^{-6} M .

Further experiments showed that thyroxine inhibited also the oxidation of DL- β -hydroxybutyrate, pyruvate, and malate. It thus appears that thyroxine

TABLE I. *Inhibition of glutamate oxidation of liver mitochondria by thyroxine and its reversal by DPN.*

Standard medium, cf. experimental part.

Exp. no.	Addition	Oxygen-uptake (μ -atoms)
1, 2	None	8.9, 10.1
	DPN	10.9, 10.6
	Thyroxine (5×10^{-5} M)	0.5, 4.3
	Thyroxine + DPN	10.0, 10.2
3	None	8.4
	Thyroxine (5×10^{-5} M)	0.4
	Thyroxine (2.5×10^{-5} M)	5.5
	Thyroxine (10^{-5} M)	6.9
	Thyroxine (10^{-6} M)	8.4

inhibits DPN-linked dehydrogenases regardless of the fact whether the enzymes are located in the soluble or insoluble part of the mitochondria [10]. Thyroxine apparently released DPN from the enzymatically-active sites of the mitochondria since DPN always reversed the inhibitions.

Effect of ethylenediaminetetraacetate, Mg^{2+} and Mn^{2+} .—Since the integrity of the mitochondria appeared to be involved in their response to thyroxine, such changes were made in the experimental conditions as are known to affect mitochondrial stability. To this end the effect of adding EDTA, varying the Mg^{2+} and adding Mn^{2+} on the glutamate oxidation of mitochondria in the presence of thyroxine, was studied.

As shown in Table II the inhibitory effect of thyroxine on glutamate oxidation was counteracted markedly or even completely when EDTA was present in a final concentration of 10^{-3} or 10^{-2} M. EDTA also counteracted the inhibition by thyroxine of the oxidations of β -hydroxybutyrate, pyruvate, and malate.

The effect of varying the Mg^{2+} concentration was studied next (Table II). Thyroxine was much more active in the low Mg^{2+} medium than in the standard medium. The oxidation of the controls was not affected when the Mg^{2+} concentration was raised to 0.0075 M, but thyroxine (5×10^{-5} M) was now less active (O_2 uptake: 2.8 μ atoms) than in the standard medium (no oxygen uptake).

It should be noted that the addition of EDTA or the lowering of the Mg^{2+} concentration affected the oxidation of glutamate by the control mitochondria

TABLE II. *Effect of EDTA and Mg^{++} on the inhibition of glutamate oxidation of liver mitochondria by thyroxine.*

Exp. no.	Addition	Standard medium	Oxygen uptake in:	
			Fortified + EDTA (10^{-3} M)	Low Mg^{++} (0.0025 M) medium
1	None (control)	8.3	8.0	—
	Thyroxine (7.5×10^{-5} M)	0.7	4.5	—
	Thyroxine + DPN	9.4	8.5	—
2	None (control)	7.5	—	7.3
	Thyroxine (5×10^{-5} M)	2.4	—	0.5
	Thyroxine (2.5×10^{-5} M)	7.2	—	4.9
	Thyroxine (10^{-5} M)	7.7	—	6.4
3	None (control)	9.3	10.8	7.9
	Thyroxine (5×10^{-5} M)	0.7	5.3	0.4
	Thyroxine (2.5×10^{-5} M)	7.2	9.8	0.9
4	None (control)	9.2	8.9 (8.5) ^a	
	Thyroxine (7.5×10^{-5} M)	1.3	6.7 (8.9) ^a	

^a EDTA 10^{-2} M.

little (exp. 3), if at all (exp. 1, 2 and 4). This was taken to mean that the biochemical integrity of the mitochondria, and the binding between DPN and the particles in particular, was much less susceptible in the absence than in the presence of thyroxine to the damage inflicted by those factors (or mechanisms) which are kept under control by EDTA and Mg^{2+} . The inverse relation between the degree of inhibition produced by thyroxine and mitochondrial integrity is further illustrated by the fact that the effect of thyroxine was always most pronounced with those mitochondria whose oxidations in the absence of thyroxine were enhanced by adding EDTA, or depressed by lowering the Mg^{2+} concentration. Mitochondria which show the latter effect are generally considered to be somewhat damaged. It was concluded from these results that thyroxine accelerated an endogenous process (latent in intact mitochondria and weakly active in slightly damaged mitochondria) which labilized the mitochondria as shown by the release of DPN.

That EDTA and Mg^{2+} affected the same basic process was shown by the fact that the thyroxine-mediated release of DPN became independent of the Mg^{2+} concentration when EDTA was present. Experiments 1 and 2 of Table III were carried out with fresh mitochondria, in exp. 3 the mitochondria

TABLE III. *Effect of EDTA on the inhibition of glutamate oxidation of liver mitochondria by thyroxine in the low Mg^{++} medium.*

Exp. no.	Addition	Oxygen-uptake in medium with:			
		Mg^{++} 0.005 M		Mg^{++} 0.0025 M	
		- EDTA (standard)	+ EDTA (10^{-3} M)	- EDTA	+ EDTA (10^{-3} M)
1	None (control)	8.6	8.5	7.9	8.2
	Thyroxine (5×10^{-5} M)	7.4	8.2	3.3	8.4
2	None (control)	9.2	9.5	9.1	9.0
	Thyroxine (5×10^{-5} M)	8.0	9.1	0.9	9.5
3	None (control)	5.8	9.3 ^a	3.6	8.6 ^a
	Thyroxine (7.5×10^{-5} M)	0.0	7.5 ^a	0.0	6.8 ^a

^a EDTA 10^{-2} M.TABLE IV. *Effect of Mn^{++} on the inhibition of glutamate oxidation of liver mitochondria by thyroxine.*

Exp. no.	Addition	Oxygen-uptake in medium with:		
		Mg^{++} 0.005 M	Mg^{++} 0.0025 M	
		(standard)	Mg^{++} 0.0025 M	Mg^{++} 0.0025 M plus Mn^{++} 0.0025 M
1	None (control)	9.8	10.0	8.0
	Thyroxine (5×10^{-5} M)	5.4	1.5	6.7
2	None (control)	10.0	9.0	7.3
	Thyroxine (5×10^{-5} M)	1.3	0.5	6.1

were damaged by washing at 15°C . In the latter case 10^{-2} M EDTA favored the oxidation of the controls and counteracted the thyroxine effect in the standard and low Mg^{2+} medium to the same extent.

Finally, as shown in Table IV, Mn^{2+} appeared to favor DPN-retention more efficiently than Mg^{2+} .

Effect of polyvinylpyrrolidone.—The biochemical integrity of isolated mitochondria is a function of their structural "intactness". Since swelling is considered to be the most prominent response of fresh liver mitochondria to thyroxine [15], the effect of PVP, a known stabilizer of mitochondrial morphology and function [30], on the thyroxine-mediated release of DPN was also studied.

TABLE V. *Effect of PVP and/or EDTA on the inhibition of glutamate oxidation of liver mitochondria by thyroxine.*

Exp. no.	Addition	Standard	Oxygen-uptake in medium:		
			plus PVP (2 %)	plus EDTA (10^{-3} M)	plus PVP+EDTA
1	None (control)	9.3	8.8	9.3	8.9
	Thyroxine (5×10^{-5} M)	0.0	3.4	7.2	7.2
	Thyroxine (2.5×10^{-5} M)	4.2	6.6	9.3	8.7
2 ^a	None (control)	4.7	3.0	14.0	11.8
	Thyroxine (5×10^{-5} M)	0.6	1.3	1.3	5.3
	Thyroxine (2.5×10^{-5} M)	0.6	1.7	3.3	10.2

^a Mitochondria washed at 15°C.

TABLE VI. *Effect of PVP on the inhibition of glutamate oxidation of liver mitochondria by thyroxine in the standard and low Mg⁺⁺ medium.*

Addition	20 minutes Standard medium	Oxygen-uptake after:	
		30 minutes Mg ⁺⁺ 0.005 M	30 minutes Mg ⁺⁺ 0.0025 M
None (control)	9.0	14.2	12.0
PVP (2 %)	7.7	12.0	10.0
Thyroxine (5×10^{-5} M)	0.3	0.4	0.3
Thyroxine + PVP	3.4	5.5	0.3
Thyroxine (2.5×10^{-5} M)	4.8	7.7	0.4
Thyroxine + PVP	6.2	8.4	0.2

Tables V and VI show the protective effect of PVP (2 per cent) against thyroxine. The polymer was relatively more efficient at the 5×10^{-5} than at the 2.5×10^{-5} M concentration of thyroxine in the standard medium. EDTA (10^{-3} M) yielded a better protection than PVP, but PVP plus EDTA was not more effective than EDTA alone. However, when the mitochondria were damaged by washing at 15°C (exp. 2 of Table V), PVP plus EDTA was more efficient than EDTA (10^{-3} M). As shown in Table VI, PVP did not protect against thyroxine at the low Mg²⁺ concentration. In none of the experiments was 7.5 per cent more effective than 2 per cent PVP.

Effect of Zn²⁺ and Cu²⁺.—The glutamate dehydrogenase is a zinc-containing enzyme [28]. Since thyroxine shows complexing properties (compare

[29]), it might inhibit the enzyme by removing or interacting with these ions. Therefore it was tried to counteract the thyroxine inhibition of glutamate oxidation by adding Zn ions. The effect of Cu^{2+} was also studied. Both Cu^{2+} and Zn^{2+} , in concentrations higher than $10^{-5} M$, inhibited the oxidation of glutamate of the control mitochondria. $10^{-5} M$ Cu^{2+} or Zn^{2+} did not alleviate the inhibition produced by various concentrations of thyroxine.

Inhibition of glutamate oxidation of mouse liver mitochondria by diethylstilboestrol.—Diethylstilboestrol ($5 \times 10^{-5} M$) inhibited the glutamate oxidation of mouse liver mitochondria completely in the standard medium at pH 7.0; at the $2.5 \times 10^{-5} M$ concentration of the hormone approximately 50 per cent inhibition was found. These inhibitions were counteracted by raising the Mg^{2+} concentration ($0.0075 M$), prevented by adding EDTA ($10^{-3} M$) and aggravated by lowering the Mg^{2+} concentration to $0.0025 M$. With $0.0025 M$ MgCl_2 plus $0.0025 M$ MnSO_4 present little, if any, inhibition was noted with $2.5 \times 10^{-5} M$ estrogen. PVP (2 and 7.5 per cent) did *not* counteract the inhibition in the standard medium. Preliminary experiments showed that the estrogen inhibited crystalline glutamate dehydrogenase (Boehringer u. S.) to a considerable extent. Mg^{2+} , Mn^{2+} , Zn^{2+} and EDTA did not counteract the inhibition of the crystalline enzyme by the estrogen.

Inhibition of glutamate oxidation of mouse liver mitochondria by o-aminoazotoluene.—As reported previously [6] $1.5 \times 10^{-4} M$ azo dye inhibits the glutamate oxidation of liver mitochondria in the standard medium at pH 7.0 for about 50 per cent; inhibitions as low as 30 and as high as 80 per cent have been found in the course of many experiments. In the presence of EDTA ($10^{-3} M$) no inhibition was noted, Mn^{2+} also counteracted but in the low Mg^{2+} medium inhibition was enhanced.

DISCUSSION

Inhibition of the glutamate oxidation of mitochondria by thyroxine has been noted previously by Lardy and Feldott [13], while Wolff and Ball [29] reported very recently that thyroxine inhibited the malate dehydrogenase in rat heart homogenates and sarcosomes. The latter work came to our attention when the present experiments were already well advanced. Wolff and Ball also briefly mention unpublished experiments in which thyroxine has been found to inhibit a number of purified DPN-linked dehydrogenases, including crystalline glutamic dehydrogenase, in a competitive manner.

In the present communication which describes the effect of thyroxine on

dehydrogenases, and in particular the glutamate dehydrogenase, of intact mouse liver mitochondria, it is shown that factors related to the general integrity of the mitochondria are involved in the response of the particles. Diethylstilboestrol too inhibits both the crystalline and mitochondrial glutamate dehydrogenase. Its effect on the mitochondrial enzyme is also counteracted by reinforcing the general stability of the particles. However, whereas PVP counteracts the thyroxine-mediated release of DPN from the mitochondrial dehydrogenase, the polymer has no effect in the case of diethylstilboestrol. Since thyroxine induces swelling of the mitochondria, whereas the estrogen does not, it may appear that swelling is at least partly involved in the release of DPN from the enzymatically-active sites of the mitochondria in the presence of thyroxine. However, though swelling often occurs concomitantly with loss of DPN [11, 12], it is in fact not known whether swelling did occur to any appreciable extent during the present experiments with thyroxine.

Hoch and Lipmann [9] have also found a depressive effect of thyroxine on DPN-linked oxidations of hamster liver mitochondria. These preparations, however, contained a suboptimal DPN-complement. Since thyroxine completely uncoupled the oxidative phosphorylation of the hamster liver mitochondria, the lack of phosphorylation may have contributed to the loss of DPN in this case. In contrast, most of our preparations from mouse liver contained an optimal DPN-complement and the oxidative phosphorylation was not impaired in the presence of thyroxine.

It is interesting that EDTA, Mg^{2+} and Mn^{2+} counteract not only the thyroxine-mediated release of DPN from the mitochondrial glutamate dehydrogenase, but also the swelling [15, 26] and the uncoupling of the oxidative phosphorylation [21, 22] caused by the hormone *in vitro*. The agreement is the more striking since Mn^{2+} is more efficient than Mg^{2+} in obliterating all three the effects of the hormone. On account of the effects of EDTA and Mg^{2+} it was concluded that in the presence of thyroxine some otherwise latent or weakly active mechanism came into play which promoted the loss of DPN from the glutamate dehydrogenase. This reasoning might thus also hold true for the two other effects of thyroxine on the mitochondria under the proper conditions. Now, EDTA is considered to protect isolated mitochondria and sarcosomes by binding endogenous Ca ions which are known to damage the morphological and enzymatic integrity (oxidative phosphorylation, DPN-retention) of the particles [24, 25]. Mg^{2+} has been shown to be necessary for maintaining mitochondrial morphology and, by doing so, to protect integrated enzymic function [1]. Mg^{2+} and Mn^{2+} protect mitochondria against the action of Ca^{2+} , and other noxious ions [3, 8, 11, 12, 17, 15]. This means that at a sub-

optimal Mg^{2+} concentration certain endogenous metals, e.g. Ca^{2+} , may have an opportunity to damage the intricate mitochondrial organization. Thyroxine appears to promote the latter effect, even at the 0.005 *M* concentration of Mg^{2+} as regards the DPN-release from the glutamate and other dehydrogenases. Accordingly, it is concluded that in the presence of thyroxine the mitochondrial organization is much more labile, i.e. susceptible to damage caused by endogenous metals such as Ca^{2+} , than in the absence of the hormone. This view is more or less in accordance with the conclusion of Beyer *et al.* [2, 4] who studied liver mitochondria from thyrotoxic rats. The prime effect of thyroxine might thus consist in rendering the mitochondrial organization extremely sensitive to Ca^{2+} or another metal ion, that is, *thyroxine accelerates the aging process*. As regards the mechanism of this effect it is of interest to note that Hunter *et al.* [11, 12] have shown that the swelling, loss of DPN, and uncoupling of oxidative phosphorylation brought about by pre-incubation of mitochondria with various ions could be prevented when the treatment was carried out anaerobically, or when Mg^{2+} , Mn^{2+} or EDTA were present during the aerobic pre-incubation phase. Since Lehninger and Ray [16] have also found that thyroxine did not induce swelling of the mitochondria under anaerobic conditions, the similarity between the effects of thyroxine and aging is very striking. The results of Hunter *et al.* suggest that certain primary degenerative changes in isolated mitochondria are of an oxidative nature catalyzed by such metals as Ca^{2+} , Cu^{2+} or Zn^{2+} . Thus one possible interpretation of the effect of thyroxine would be that the hormone labilizes the mitochondrial structure by favoring this unknown oxidative reaction via an endogenous trace metal.

Regarding the *in vivo* effect of thyroxine, or its active principle, it is of significance that Maley and Lardy [18] have found that with ferricyanide as electron acceptor, the oxidation of β -hydroxybutyrate of liver mitochondria from thyrotoxic rats is markedly enhanced by the addition of DPN, and that the DPN-content of these particles is 40 per cent below the norm.

SUMMARY

1. The inhibitory effect of thyroxine on several DPN-linked dehydrogenases of mouse liver mitochondria has been studied. Since the inhibitions were prevented in the presence of DPN, it was concluded that the hormone released the coenzyme from the enzymatically-active sites.

2. EDTA, Mg^{2+} , Mn^{2+} and PVP counteracted or prevented the thyroxine-mediated release of DPN from the mitochondrial glutamate dehydrogenase.

3. The results lend additional support to the view that thyroxine labilizes the functional integrity of the mitochondria rather than acting upon phosphorylation in a specific way.

4. Diethylstilboestrol was found to inhibit both the mitochondrial and crystalline glutamate dehydrogenase. The inhibition of the mitochondrial enzyme could be overcome or counteracted by adding DPN, EDTA, Mn^{2+} or Mg^{2+} , but not by PVP.

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THE INFLUENCE OF HORMONES ON CELL DIVISION

II. TIME RESPONSE OF EAR, SEMINAL VESICLE, COAGULATING GLAND AND VENTRAL PROSTATE OF CASTRATE MALE MICE TO A SINGLE INJECTION OF TESTOSTERONE PROPIONATE¹

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A PREVIOUS report has described the time-course mitotic response of seminal vesicle, coagulating gland, ventral prostate and ear epidermis to a single injection of estradiol benzoate [1]. This paper deals with the time-course mitotic response of these same tissues to a single injection of testosterone propionate.

MATERIALS AND METHODS

The methods employed in this investigation are identical to those used in prior work [1]. Testosterone propionate (Schering) has been administered as a single subcutaneous injection of 16 micrograms in 0.25 cc sesame oil (N.F.). Statistical comparison of data has been made by application of standard methods.

RESULTS

Comparison of mitotic indices obtained in tissues from intact control animals with those obtained in tissues from castrate control animals (Table I) indicates that orchidectomy results in a depression of mitotic activity in all cases. The *T*-test shows this drop to be significant in ear, coagulating gland and ventral prostate ($P < 0.01$) but not significant in the case of seminal vesicle ($P = 0.4$).

The response of ear epidermis to testosterone propionate is presented graphically in Fig. 1. Significant increases in mitotic activity over castrate levels are noted only during the periods 18-24 hours and 36-42 hours ($P < 0.02$) following hormone administration. Inspection of this curve reveals a series of fluctuations which have an apparent period of 18-30 hours.

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TABLE I. *Mitotic activity at six-hour intervals following the injection of 16 μ g testosterone propionate into castrate male mice.*

For each tissue mean mitotic indices as log (per mille mitotic index $\times$ 100) followed by standard error values are given. The number of donor mice for tissues examined follows in brackets.

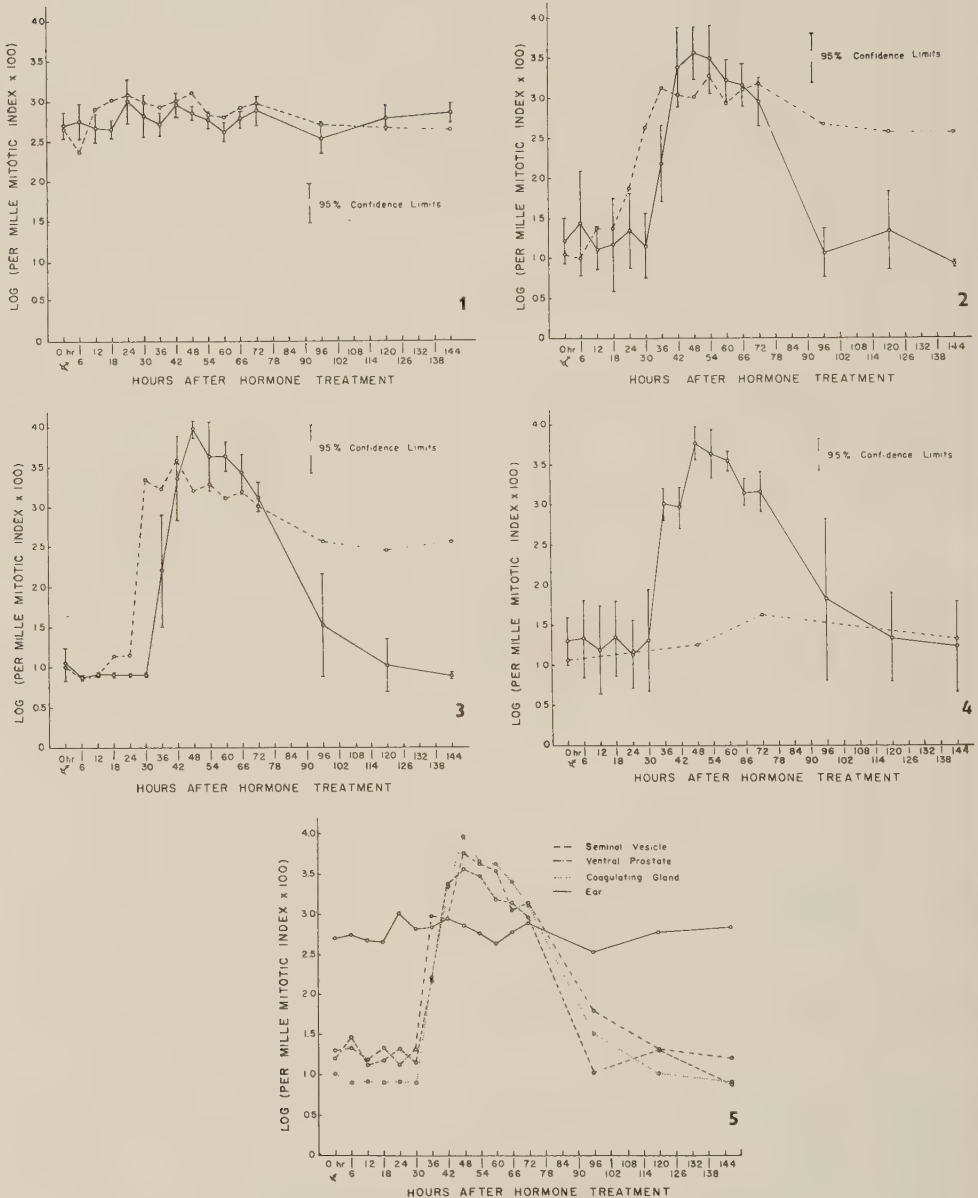
Sample category	Ear	Seminal vesicle	Coagulating gland	Ventral prostate
Normal	2.910 0.033 (10)	1.328 0.119 (10)	1.860 0.052 (10)	2.060 0.064 (10)
Castrate	2.702 0.062 (9)	1.208 0.123 (9)	1.034 0.088 (9)	1.301 0.127 (9)
0-6 hrs.	2.744 0.073 (5)	1.486 0.235 (5)	0.897 0.006 (5)	1.335 0.180 (5)
6-12	2.673 0.070 (6)	1.110 0.106 (6)	0.905 0.005 (6)	1.190 0.199 (5)
12-18	2.656 0.037 (5)	1.187 0.210 (5)	0.093 0.007 (5)	1.325 0.178 (5)
18-24	3.040 0.105 (5)	1.338 0.179 (5)	0.903 0.002 (5)	1.144 0.146 (5)
24-30	2.822 0.096 (5)	1.154 0.145 (5)	0.897 0.036 (5)	1.305 0.231 (5)
30-36	2.716 0.068 (7)	2.179 0.181 (6)	2.201 0.252 (5)	2.999 0.081 (7)
36-42	2.957 0.051 (5)	3.376 0.185 (5)	3.367 0.185 (5)	2.957 0.094 (5)
42-48	2.835 0.026 (5)	3.554 0.117 (5)	3.971 0.038 (5)	3.774 0.071 (5)
48-54	2.757 0.035 (5)	3.480 0.132 (5)	3.616 0.135 (4)	3.625 0.099 (4)
54-60	2.608 0.044 (6)	3.203 0.093 (5)	3.620 0.071 (6)	3.555 0.039 (5)
60-66	2.776 0.040 (9)	3.146 0.106 (7)	3.404 0.099 (7)	3.135 0.068 (7)
66-72	2.877 0.071 (5)	2.952 0.105 (5)	3.112 0.067 (5)	3.158 0.098 (5)
90-96	2.522 0.070 (5)	1.036 0.119 (5)	1.507 0.210 (4)	1.806 0.316 (4)
114-120	2.765 0.057 (5)	1.338 0.180 (5)	1.026 0.121 (5)	1.313 0.193 (5)
138-144	2.840 0.028 (5)	0.911 0.002 (5)	0.909 0.035 (5)	1.215 0.192 (5)

Analysis of variance between 0-72 hours indicates that these points form a heterogeneous series ($P < 0.01$) thus suggesting the fluctuations to be real.

The response of seminal vesicle epithelium is presented graphically in Fig. 2. A significant increase in mitotic activity above castrate levels is noted during the period 30-36 hours following hormone administration ($P < 0.01$). Peak activity is reached 42-48 hours after commencement of treatment. Mitotic activity has returned to castrate levels 96 hours after beginning of hormone treatment ($P > 0.10$).

Development of mitotic activity in the epithelium of coagulating gland is presented graphically in Fig. 3. Mitotic activity rises significantly above castrate levels during the period 30-36 hours after hormone injection ($P < 0.01$). Peak activity is developed 42-48 hours following treatment. Mitotic activity has returned to castrate levels at 120 hours ($P > 0.10$).

The response of ventral prostate epithelium is represented graphically in Fig. 4. Significant increase in mitotic activity in this tissue is noted during the period 30-36 hours after hormone administration ($P < 0.01$). Peak ac-



Figs. 1-5.—Time response of epithelial cells to a single injection of $16 \mu\text{g}$ of testosterone propionate (solid line) and to a single injection of $16 \mu\text{g}$ of estradiol benzoate (1) (dashed line). Ninety-five per cent confidence limits are indicated by vertical lines. Each point on the abscissa (X) indicates the six-hour period during which mitoses were arrested by colchicine, and the actual time period involved is (X-6) to X hours. Fig. 1. Time response of ear Malpighian layer epithelial cells. Fig. 2. Time response of seminal vesicle epithelial cells. Fig. 3. Time response of coagulating gland epithelial cells. Fig. 4. Time response of ventral prostate epithelial cells. Fig. 5. Time response of the epithelial cells of Malpighian layer of ear, acinar epithelial cells of seminal vesicle, coagulating gland and ventral prostate.

tivity is reached 42-48 hours after institution of treatment. Mitotic activity has returned to castrate levels at 96 hours ($P > 0.10$).

The responses of all tissues examined are plotted on the same set of coordinates in Fig. 5. Examination of the data presented in this fashion emphasizes certain points. (a) All tissues exhibit a latent period before increased mitotic activity is evident. This latent period is constant in the case of seminal vesicle, coagulating gland and ventral prostate and is 30 hours in length. The latent period of ear epidermis is 18 hours. (b) Seminal vesicle, coagulating gland and ventral prostate reach peak mitotic activity 48 hours after hormone administration. The level of activity reached at this time by coagulating gland is significantly higher than that exhibited by seminal vesicle ($P < 0.02$). Ventral prostate is intermediate in response ($P > 0.05$ *vs.* seminal vesicle and coagulating gland). Seminal vesicle, coagulating gland and ventral prostate reach significantly higher levels of activity (48 hours) than ear epidermis (24 or 42 hours) ($P < 0.01$).

DISCUSSION

Comparison of the above results with those obtained following administration of estradiol benzoate [1] is of interest.

The response of ventral prostate epithelium to estradiol benzoate is in marked contrast to the response obtained with testosterone propionate (Fig. 4). This tissue shows no significant increase in mitotic activity until 72 hours after administration of estradiol benzoate. Testosterone propionate, however, elicits a significantly greater mitotic response (by inspection) and does so within 30-36 hours after hormone treatment. The response following treatment with this androgen is about 100 times greater than that obtained with estradiol benzoate.

The response of coagulating gland epithelium to estradiol benzoate and testosterone propionate is compared graphically in Fig. 3. Inspection of this figure indicates that the latent period is different under the two treatment conditions. With testosterone propionate it is 30 hours in contrast to 24 hours with estradiol benzoate. Testosterone propionate elicits greater mitotic activity than does estradiol benzoate ($P < 0.01$; estradiol benzoate 42 hours *vs.* testosterone propionate 48 hours). Mitotic activity under estrogen treatment is more protracted than under androgen treatment. Activity following administration of estradiol benzoate remains significantly above castrate levels 144 hours after hormone treatment but with testosterone propionate has returned to castrate levels at 120 hours.

The response of seminal vesicle epithelium to estrogen and androgen is compared graphically in Fig. 2. Examination of this figure indicates that the latent period following administration of estradiol benzoate is 18 hours as opposed to 30 hours with testosterone propionate. The levels of mitotic activity reached under the two treatment conditions are equivalent ($P > 0.10$; testosterone propionate 48 hours *vs.* estradiol benzoate 54 hours; testosterone propionate *vs.* estradiol benzoate between 42 and 66 hours). Mitotic activity remains significantly higher than castrate levels for 144 hours following estradiol benzoate administration in contrast to the return to castrate levels at 96 hours following testosterone propionate treatment.

The mitotic activity evoked by androgen and estrogen in ear epidermis is represented graphically in Fig. 1. It will be noted that the latent period under estrogen treatment is 6 hours while under androgen treatment the latent period appears to be 18 hours. The mitotic response of this tissue to estradiol benzoate is significantly greater than the response to testosterone propionate ($P < 0.001$ between 24–72 hours).

From the above discussion it appears that the responses of the investigated tissues to testosterone propionate and estradiol benzoate differ in several respects. First, the latent period observed under estrogen treatment is variable while under androgen treatment it is constant at least in the case of seminal vesicle, coagulating gland and ventral prostate. Second, under estrogen treatment the time at which seminal vesicle, coagulating gland and ventral prostate reach peak activity is variable while under androgen treatment it is constant. Third, the level of mitotic activity reached following administration of these hormones differs significantly in the case of ear and ventral prostate. Ear epidermis is more responsive to estradiol benzoate while ventral prostate is more responsive to testosterone propionate. Seminal vesicle and coagulating gland respond nearly equally to both hormones. Fourth, return of mitotic activity to castrate levels in seminal vesicle, coagulating gland and ventral prostate is more rapid following testosterone propionate treatment than following estradiol benzoate treatment.

The time-course mitotic response of seminal vesicle and ventral prostate reported above compares favorably with the time-course mitotic response of these same tissues under androgen stimulation in the rat [4]. Thus, in the rat these tissues respond significantly 35 hours following hormone administration and reach their maximal activities at about 48 hours. The return to castrate mitotic levels is comparable in the two species and occurs in the rat about 100 hours after commencement of treatment.

The difference in response of the ventral prostate epithelium to testosterone

propionate and estradiol benzoate appears important in terms of the mechanism through which these hormones exert their effects on mitotic activity. Currently, it is common to assume that hormonal materials exert their characteristic effects by an influence on enzyme systems. Interpreted in this fashion the differential response of ventral prostate epithelium to androgen and estrogen suggests that these hormones exert their mitogenic actions through an influence upon different metabolic systems. The slight mitotic response elicited in the epithelium of ventral prostate by estradiol benzoate suggests that an estrogen sensitive metabolic step which is critical in terms of mitotic activity may be absent in this tissue. This step, of course, is present in those tissues responding mitotically to estrogen administration (e.g. seminal vesicle, coagulating gland or ear). Evidence in the literature suggests that one primary enzymatic reaction affected by estrogenic materials is the hexokinase reaction. Thus, Bullough [2] finds that estrogen treatment significantly enhances the mitotic activity of ear epidermis (measured *in vitro*) in the presence of glucose but has no enhancing effect when fructose or pyruvate are supplied. These results were interpreted as indicating the hexokinase reaction to be rate limiting in terms of estrogen-stimulated cell division in ear epidermis. Biochemical evidence also suggests that the hexokinase reaction is stimulated by estrogenic materials [9]. No information is available concerning the response of this enzyme in rodent ventral prostate to estrogen.

Seminal vesicle and ventral prostate of the rat may show decreased levels of oxidative metabolism following orchidectomy [6, 8]. Administration of androgen reverses this change [6, 8]. The activity of the cytochrome oxidase, succinic dehydrogenase, aconitase, fumarase and malic dehydrogenase systems in these tissues is depressed by castration [5, 10]. Administration of androgen reverses the changes in the activity of these enzymes [5, 10]. These facts indicate that androgens may affect primarily the reactions of the tricarboxylic acid cycle. If this assumption is correct it might be expected that the enzymatic steps which are critical in the development of mitotic activity under androgen treatment lie in this metabolic area. No direct evidence bears on this question. However, Bullough [3] has shown that testosterone treatment does not enhance the ability of glucose to maintain mitotic activity in ear epidermis (measured *in vitro*). This fact suggests that the metabolic points of androgenic stimulation of mitotic activity lie distal to the preliminary phosphorylation of glucose.

One time-course study in the literature presents data on the development of oxidative metabolism in secretion-free rat seminal vesicle following androgen treatment [7]. Q_{O_2} values under these conditions reached maximal levels 48

hours after commencement of hormone treatment and then declined to control levels even under continued hormone administration. The correlation of this increase in Q_{O_2} level with development of mitotic activity is apparent.

SUMMARY

The development of mitotic activity following administration of a single dose of testosterone propionate has been followed in the epithelia of ear, seminal vesicle, coagulating gland and ventral prostate of castrate male mice. All tissues respond to androgen treatment by significantly increased mitotic activity. Ear epidermis shows significantly increased activity 18 hours following hormone treatment while all other tissues show significantly increased activity 30 hours after treatment. Seminal vesicle, coagulating gland and ventral prostate reach peak activity 48 hours following hormone injection. Mitotic activity in these tissues has returned to castrate levels 96–120 hours after commencement of treatment. The response of these tissues to testosterone propionate is compared with the response to estradiol benzoate [1]. The results are discussed in terms of hormonal effects upon metabolic systems which may be critical in the development of mitotic activity.

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HORMONAL INFLUENCE ON THE NUCLEIC ACID AND PROTEIN CONTENTS OF THE HUMAN MYOMETRIUM

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HORMONAL induction of uterine growth is a well-known phenomenon. The effect is pronounced in all the different parts of the organ, but from a quantitative point of view the growth response of the myometrium dominates the picture. The mechanism whereby the trigger substance brings about its action in the target organ is completely obscure. Very few quantitative studies on the cellular processes underlying and accompanying the growth of this organ have been carried out, and no data on human material are available.

The important role of the nucleic acids in connection with processes of growth has long been realized. Intimate relationships have been supposed to exist between protein synthesis, the quantitatively dominating process during growth, and nucleic acid metabolism [6, 8, 14, 21, 28]. Recently strong experimental evidence has been brought forward in support of this hypothesis [15, 16, 17, 19, 34].

This paper is a report on studies on the nucleic acid and protein contents in the myometrial part of the human uterus. The investigation was planned with special emphasis on the effect of the ovarian hormones, and uteri under the influence of various hormone levels have been investigated. Specimens have been analyzed from uteri of women in the post-menopausal period (post-menopausal group), from non-pregnant women with existing ovarian activity (non-pregnant group) and from pregnant women at different stages of pregnancy (pregnant group).

MATERIAL AND METHODS

Human myometrium from the fundus of the uterus was obtained from patients operated upon for uterine myoma, cysts of the ovaries and for sterilization after parturition. During pregnancy, material was obtained in connection with legal abortions and caesarean sections. In the last-mentioned operations material was taken from the fundus in one case and from the lower part of the corpus in the two other

cases. When performing hysterectomies the specimens were excised before the uterine arteries were clamped. The endometrium and mesometrium were cut off and the piece was immediately (within 30–40 seconds) put into a beaker immersed in dry ice.

The phase of the cycle was determined by microscopic investigation of an endometrial biopsy.

The tissues were homogenized in a 5 per cent trichloroacetic acid solution with a Bühler homogenizer at 40,000 r.p.m. for four minutes. The tissue concentration was about 10 per cent. Precautions were taken to keep the temperature close to 0°C.

The nucleic acids were extracted according to the method of Schneider [30].

*Determination of PNAP.*¹—One volume, containing the nucleic acid to be analyzed, is chilled in an ice-water bath. Four volumes of concentrated chilled (–15°C) H₂SO₄ are added, the acid being allowed to run along the tube wall. After mixing, the glass-stoppered tube is placed in a boiling waterbath for 15 minutes. After cooling to room temperature, 0.1 ml of 3 per cent cysteine-HCl (w/v) is added with an Agla micro-meter syringe (Burroughs Wellcome & Co., London) and the contents are mixed. The tube is placed in a water bath at 25°C for 20 minutes. The optical densities are read at 390 and 428 $\mu\mu$ with a blank consisting of tissue extract and H₂SO₄. The difference between the extinctions at these two wavelengths is proportional to the amount of PNAP. As a standard of reference, PNA, extracted from yeast according to the method of Crestfield, Smith and Allen [9], was used. One μg PNAP gives an $E_{390} - E_{428}$ value of 0.073. For further details the reader is referred to Brody [4].

DNAP was determined as described earlier [5].

After nucleic acid extraction the tissue residue was washed several times with distilled water. The lipides were then extracted according to Schneider [30]. The residue was dried to constant weight and the dry weight determined.

The colorimetric determinations were carried out in a Beckman DU quartz spectrophotometer with a 1 cm cell. Centrifugations were performed in a M.S.E. refrigerated centrifuge.

RESULTS AND DISCUSSION

A systematic approach to the study of the cellular processes connected with the growth of an organ requires the fulfilment of certain conditions. The tissue to be studied must be available in sufficient quantity, must possess a pronounced growth intensity and must be of homogeneous cellular composition and distribution. Human myometrium fulfils all these requirements.

The uterus is a target organ for the estrogens and the growth-inducing capacity of this group of hormones and their reciprocal interaction have been clearly demonstrated by Hisaw, Velardo and Goolsby [22]. The hormonal induction of growth shows the problem under a fascinating aspect. It enables one to study the normal response of the target organ to the trigger substance, to chart the biochemical changes accompanying the normal growth and

¹ Abbreviations: DNA, deoxypentose nucleic acid; DNAP, DNA-phosphorus; PNA, pentose nucleic acid; PNAP, PNA-phosphorus.

TABLE I. *Changes in nucleic acid and protein contents of human myometrium.*

State of uterus	μg DNAP per mg dry weight	Number of nuclei per mg dry weight $\times 10^{-6}$	pg^a dry mass per tissue unit ^b	pg^a PNAP per tissue unit ^b
Post-menopausal	4.75	7.37	135.71	0.37
	3.67	5.66	176.59	0.41
Non-pregnant				
menstruation	1.74	2.67	374.21	0.70
6th day	1.69	2.60	383.87	0.81
8th day	1.36	2.09	479.21	0.59
18th day	1.45	2.23	447.42	0.81
19th day	1.62	2.49	401.35	0.73
21st day	1.43	2.20	455.21	0.88
23rd day	1.56	2.40	416.56	0.98
26th day	1.78	2.73	366.52	0.96
Pregnant				
17th week	0.79	1.22	819.65	2.03
20th week	0.57	0.88	1140.10	2.81
40th week	0.24	0.37	2726.72	4.64
40th week	0.30	0.46	2162.96	5.26
Puerperal				
4th day after parturition	0.43	0.66	1511.31	2.24

^a 1 $\text{pg} = 10^{-12}$ g.^b Calculated on the assumption of an average DNAP content per nucleus of 0.65 pg (see text).

differentiation in this tissue, and to characterize in terms of quantitative chemical data the deviations from the normal response [7].

The modifications of the PNAP determination procedure [4] introduced here enhance the sensitivity, accuracy and simplicity of the method. The sensitivity is increased by about 15 per cent, the accuracy is considerably increased, the error of the method now being 1.6 per cent. A practical drawback of the original method was the necessity to perform the optical density readings at an exact time after the addition of cysteine-HCl, which put a limit to the number of assays which could be performed at the same time. With the present modifications the optical densities are stable for a long period.

A striking result of this investigation is the pronounced changes in the amount of DNAP per mg dry weight. The values are given in Table I. As

is seen, the DNAP-content per mg dry weight is highest in the post-menopausal uterus. Hormonal activity brings about a decrease, moderate in the non-pregnant uterus and considerable in the pregnant one.

The discovery by Boivin, Vendrely and Vendrely [2] that the average DNAP-content per somatic nucleus is constant in a given species, and double that of the sperm cell, has been repeatedly confirmed [11, 12, 25, 26]. In a wide variety of material the average amount of DNAP per nucleus has been found to be 0.65 pg (1 pg = 10^{-12} g) [6, 33]. This quantitative constancy of a nuclear constituent has widely ramifying biochemical implications. It permits, among other things, the calculation of the number of nuclei in a tissue under study. Table I gives the figures in the different uteri analyzed.

Figs. 1-4 show photomicrographs of stained sections from four uteri at different stages of ovarian hormone influence and uterine growth. The nuclear density is impressive in the post-menopausal uterus, somewhat less pronounced in the non-pregnant uterus, and considerably decreased during pregnancy. The pictures clearly illustrate the biochemical data.

The human uterus undergoes considerable growth during pregnancy. Gillespie [18] has reported some data on the increase in weight. He found a uterus at the end of pregnancy to weigh 30 times more than a non-pregnant uterus. To some extent the growth of the organ is due to hyperplasia (cell division). This form of cellular growth is generally considered to be limited to the early periods of gestation [29]. Hypertrophy (increase in cell volume) is the other factor of uterine growth. The increase in the size of the muscle cell implicates the whole cell, including the nucleus [29, 31, 32]. The increase in cytoplasmic volume, however, is considerably greater than that of the nucleus. These differences in the growth of different parts of the cell bring about profound changes in the nuclear/extranuclear volume ratio. This is the morphological basis of the biochemically registered decrease in nuclear density observed during maturation and gestation.

The results presented here demonstrate that the tissue composition and structure are profoundly changed during the process of growth. Under the assumption that the water content is approximately the same in these different states, as, actually, it has been shown to be [10, 23], it is clear that in the uterus the average amount of protein per cell (i.e. the dominating tissue constituent) exhibits large fluctuations in varying conditions as for instance in the castrate, in the mature organism and during pregnancy. This finding brings us to the question of an acceptable standard of reference for cellular constituents, especially when these are studied during the growth and development of an organ. As a rule the results of tissue analyses have been referred to

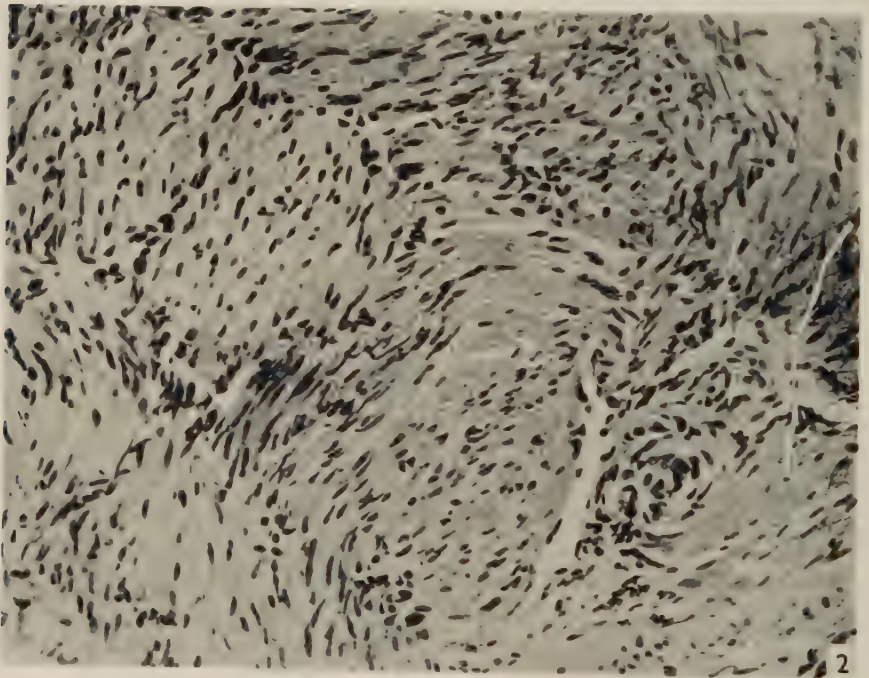
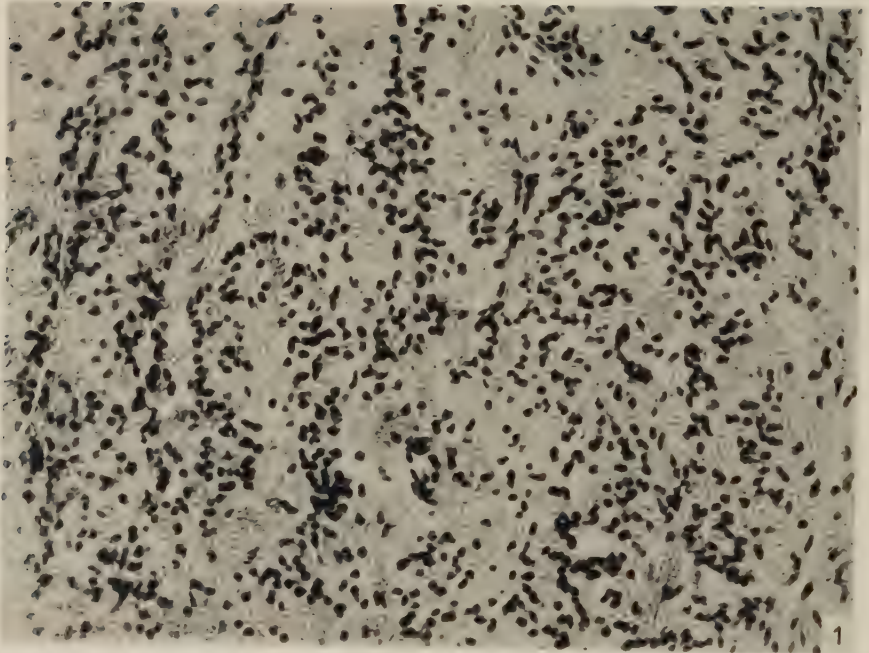
wet or dry weight, i.e. to the protein content of the tissue. This standard, however, is itself a variable, showing a close correlation to the growth process (see below).

A biochemically more precise approach is to express the tissue constituent on a per nucleus basis [11, 12]. When one applies this method to the quantitative calculation, the results are not obscured by changes in the standard of reference and even rather subtle physiological and pathological changes in the cell may be revealed. Table I shows the considerable increase in PNAP per tissue unit during maturation and pregnancy. The expression "tissue unit" is used in the sense of a statistical unit and implies the average amount of extracellular and intracellular tissue per nucleus. It is seen that hormonal influence, as this is exercised during the period of ovarian activity, increases the PNAP-content approximately two to three times, as compared with the value found in a post-menopausal uterus. During pregnancy there is a considerable accumulation of PNAP in the cell and a continuous increase towards term. During the first 20 weeks there is a proportionally much more rapid increase, coinciding with the period of rapid growth as judged by the weight increase of the uterus [18]. Immediately after parturition there is a sharp drop in the PNAP-content.

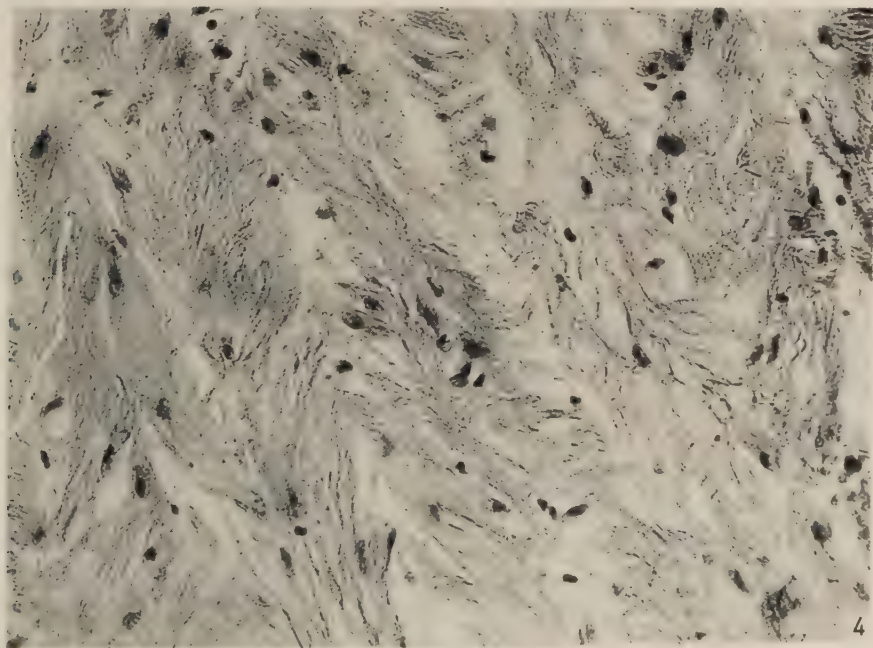
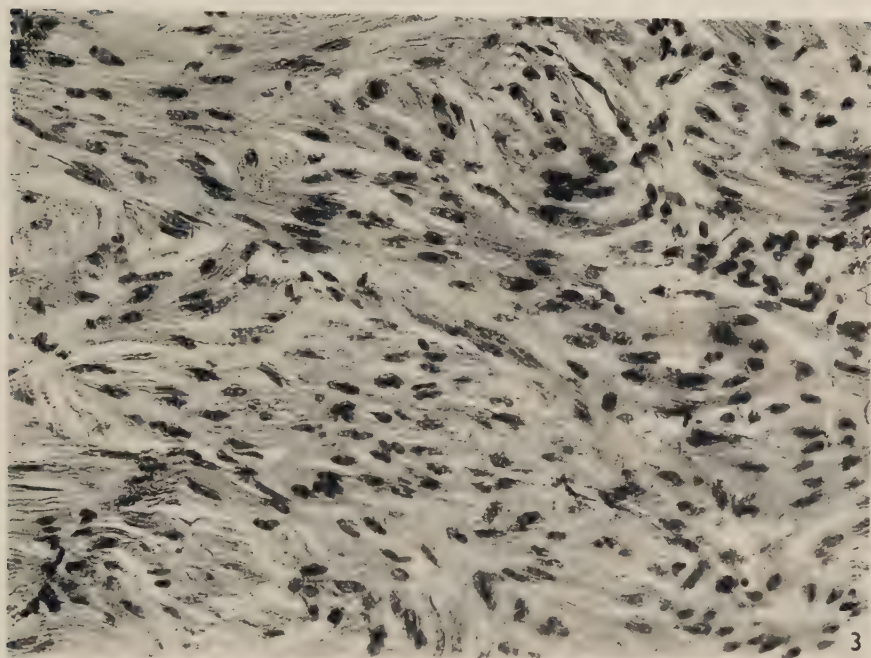
There has been much speculation on the problem of the relative importance of cellular hypertrophy and hyperplasia in the mechanism of uterine growth during pregnancy. For a detailed discussion from a morphological point of view the reader is referred to Salvatore [29]. The biochemical findings reported here indicate the occurrence of both types of growth to about the same extent. As is seen in Table I, the dry mass per tissue unit is approximately 6-fold increased from the non-pregnant state to the term of pregnancy. As the water content is approximately the same at these different periods [10, 23], this implies that growth by cellular division accounts for about a 5-fold increase in the uterine weight. This calculation is based on the data of the weight increase of the human uterus given by Gillespie [18].

When investigating a tissue constituent synthesized and strictly localized within the cell, reference to the tissue-DNAP brings the analytical results down to a cellular level. The average amount per cell may be calculated, as has been done with the PNAP in Table I. Reference is hereby made to a well-defined morphological and functional unit of the tissue, tissue unit in this case being equivalent to cell.

It should be pointed out, however, that dry mass per tissue unit is not equivalent to dry mass per cell. Dry mass in this investigation refers to extracellular as well as intracellular protein. In this case, too, the same stan-



Figs. 1-4.—Sections from uteri at different physiological states stained with haematoxylin and eosin and reproduced at an original magnification of $\times 250$. *Fig. 1*, post-menopausal uterus. *Fig. 2*, nonpregnant uterus. *Fig. 3*, 20th week of pregnancy. *Fig. 4*, 40th week of pregnancy.



dard of reference is to be recommended. It refers the protein content to a functional unit. This view is based on the presumption that the extracellular protein is also synthesized within the cell. In this connection an interesting study by Needham and Cawkwell [27] should be mentioned. These authors investigated the protein composition and nucleic acid content of the rat uterus in different states and separated the protein into four fractions, including actomyosin-like protein (intracellular) and collagen (extracellular). The data are presented on a wet weight basis. A recalculation by the present writer with reference to the DNAP indicates a somewhat more than 6-fold increase of actomyosin-like protein from the castrated state to the end of pregnancy. The collagen, on the other hand, is only somewhat more than doubled under the same conditions.

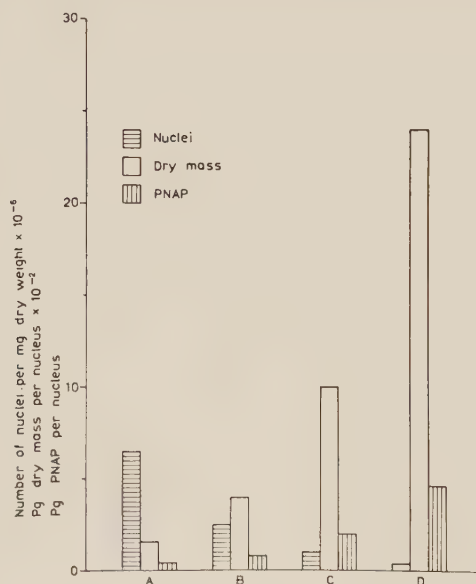
DNA is a constant structural component of the chromosome and is not found in non-chromosomal cell structures. It is therefore obvious that during hyperplastic growth there is a phase in the mitotic cycle when chromosomal reproduction and the concomitant synthesis of new DNA are occurring, but division of the nucleus and the cell has not yet taken place. The cells which enter the mitotic cycle will thus contain different amounts of DNA, from the ordinary average content of a somatic nucleus up to twice this amount. These considerations make it conceivable that the process of intense cellular division may cause an increased average DNA-content per nucleus.

Comparatively little attention has been paid to this problem as it is presented during the normal growth of an organ. However, in a study on the growth of the human placenta [6] nuclei were isolated from placentas at different stages of development, their number counted, and the average DNA-content per nucleus determined. Only a small decrease was found in placentas of widely differing growth intensities. This investigation thus justifies the conclusion that even a rather intense process of nuclear multiplication as this occurs during the normal growth of an organ, will have only a comparatively small influence on the average DNA-content of the cell population. Changes in this value will probably reflect the mitotic frequency. The results arrived at by Thomson, Heagy, Hutchison and Davidson [33] support this hypothesis. These authors found the average DNAP-content per nucleus during the first four days after partial hepatectomy in the rat to be 10–50 per cent above the mean value found for unoperated animals. Mitosis is reported to occur with a frequency of 1 in 7000 to 1 in 20,000 in the liver of a normal adult rat [24], while it may reach values of 1 in 40 for parenchymal cells after partial hepatectomy [1].

In the light of the above considerations it may be said that the results

given here express on the cellular level some quantitative changes in the human myometrium during hormonally induced growth. The material presented may be regarded as a series of target-organ specimens from the stage of absent or minimal trigger-substance influence (post-menopausal

Fig. 5.—Survey of changes in nucleic acid and protein contents in the human myometrium. State of uterus: A, post-menopausal; B, non-pregnant; C, pregnant 20th week; D, pregnant 40th week. The expression "per nucleus" is identical with per tissue unit. For further explanation, see text.



uterus) to the stage of maximal influence (term of pregnancy). The figures given in Table I express the average net synthesis of PNA and protein per nucleus during this growth process. It is interesting to note that the relative increase of protein and PNA per tissue unit is approximately the same, being about 15 times for the protein and 12 times for the PNA. A graphical survey of these results is presented in Fig. 5.

Since in all the different states subjected to ovarian hormone influence, with the possible exception of the proliferative phase of the menstrual cycle, estrogens as well as progesterone are secreted, it is not possible to draw any conclusions from this study as to the relative effect of either group of hormones. Experiments on animals have, however, shown the estrogens to be the prime factor in the growth of the uterus [22]. Injection of estradiol-benzoate has been found to increase the amount of PNA in the myometrium of the rabbit [3] and the rat [13]. On the other hand, administration of estradiol-benzoate as well as progesterone increases the turnover-rate of PNAP in the rabbit myometrium [3].

SUMMARY

The protein and nucleic acid contents in human myometrium from women in the post-menopausal period, from non-pregnant women with ovarian function and from pregnant women at different stages of pregnancy have been determined.

The deoxypentose nucleic acid content per mg dry weight is very high in the post-menopausal group, reflecting a considerable nuclear density per unit tissue in this state. Ovarian hormone activity causes a moderate decrease in the density in the non-pregnant group and a considerable reduction during pregnancy. Concomitant and reversely proportional to these changes are the findings of small amounts of protein per tissue unit in the post-menopausal uteri, moderately increasing values in the non-pregnant state and relatively very high values during pregnancy.

The pentose nucleic acid content per tissue unit is very low in the post-menopausal group, increases under the influence of ovarian hormones and exhibits very high values during pregnancy. The relative increase during the first 20 weeks of pregnancy is more pronounced than after this period. This finding indicates that the main accumulation of PNA in the cell coincides with the period of rapid growth as judged from the weight-increase of the human uterus.

During the normal process of hormone-induced growth, represented by this material, the relative increase in protein and PNA per tissue unit is approximately the same, being about 15 times for the protein and 12 times for the PNA.

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NUCLEOTIDE AND NUCLEIC ACID METABOLISM IN DEVELOPING AMPHIBIAN EMBRYOS

I. ISOLATION AND CHEMICAL IDENTIFICATION OF ACID-SOLUBLE NUCLEOTIDES¹

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THE developing amphibian embryo presents a unique opportunity for studying phosphate and energy transfer in an essentially closed system. Administration of radioactive phosphorus to the female frog leads to incorporation of the isotope into the nucleotides and nucleic acids of the eggs and, once the eggs are shed from the body, they are not further exposed to the isotope present in the adult. This means that the complicating factor of having the eggs exposed to the phosphate pool of the adult during the experimental period is eliminated. Any exchange of radioactive phosphate which occurs takes place within the egg only.

With this in mind, a broad study of the phosphate transfer in the acid-soluble nucleotides and nucleic acids during the development of the amphibian egg is now underway in our laboratory. The first report of this study is concerned with the isolation and chemical identification of the acid-soluble nucleotides found in frog eggs and embryos.

Isolation of the Acid-soluble Nucleotides

Materials and Methods.—Adult *Rana pipiens* females were injected intraperitoneally with 0.5 $\mu\text{c/g}$ of body weight of P^{32} as sodium phosphate and with whole pituitary glands [3]. A mature, labeled, unfertilized sample of at least 500 eggs was thus obtained and manually stripped of jelly. The unfertilized samples were placed in about 20 ml of ice-cold 5 N perchloric acid and homogenized with a glass tissue grinder. After centrifugation in the cold, the supernatant fluid was removed and the precipitate washed twice with cold 0.6 N perchloric acid. The washings were pooled with the first supernatant and the entire acid-soluble fraction was carefully neutralized to pH 6.5 with 5 N KOH in the cold. The insoluble potassium perchlorate was eliminated

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by centrifugation. The chromatographic procedure of Hurlbert *et al.* [4] was followed to separate the nucleotides on a Dowex-1-formate column using gradient elution. The samples were collected in an automatic fraction collector and were read at 260 and 280 $m\mu$ using a Model DU Beckman spectrophotometer. When necessary, the samples were further purified by paper or column chromatography or by paper electrophoresis. In the preliminary investigations of P^{32} incorporation reported here, a 1.0 ml volume of each fraction was removed, plated, and counted using a thin mica-window Geiger tube.

Chemical Identification of the Acid-soluble Nucleotides

The same ultraviolet-absorbing peaks from several clutches of eggs were pooled to obtain a sufficiently large sample for chemical identification. The pooled fractions from the column were dried *in vacuo* using a series of traps containing $CaCl_2$ layered with NaOH. The traps were immersed in ice-water baths and the desiccator, containing the samples, was carefully warmed with an infra-red lamp to facilitate the removal of the ammonium formate. The dried samples were redissolved in glass distilled water.

Ultraviolet absorption analysis.—Each sample was adjusted to 0.01 *M* with respect to HCl and the ultraviolet absorption spectrum recorded. The concentrations of the samples were calculated using the following millimolar extinction coefficients [4] at the wavelengths of maximum absorption: cytidine, 13.7 at 278 $m\mu$; adenosine, 14.1 at 258 $m\mu$; guanosine, 13.3 at 265 $m\mu$; and uridine, 9.9 at 260 $m\mu$.

Pentose analysis.—To 1.0 ml aliquots of each sample 2.0 ml of freshly prepared orcinol reagent were added. The reagent was prepared by dissolving 100 mg of $FeCl_3 \cdot 6 H_2O$ in 100 ml of concentrated HCl and adding 3.5 ml of a 6 per cent solution of orcinol in ethanol [2]. The samples were capped, heated for 15 minutes in a boiling water bath, cooled, and read at 665 $m\mu$. A 0.5 mM solution of adenosine-5'-phosphate was used as a standard.

Phosphate analysis.—Inorganic, labile, and total phosphate were determined on each sample using the stannous chloride method [6] with the following modifications: Total phosphate: 0.1 ml of each sample was digested with 1.0 ml of 5 per cent H_2SO_4 . After digestion and clearing with one drop of 1:1 perchloric acid, the sample was brought to 2.0 ml with water and the color developed as in the inorganic and labile phosphorus determinations. Labile phosphate: to a 0.1 ml volume of sample which was brought to 1.0 ml with water, 1.0 ml of 4 per cent H_2SO_4 was added. The samples were capped and heated for 15 minutes in a boiling water bath. After cooling the color was developed. Inorganic phosphate: to 0.1 ml volume of sample which had been brought to 1.0 ml with water, 1.0 ml of 4 per cent H_2SO_4 was added. Each total, labile and inorganic phosphate sample then received 1.0 ml of 0.12 per cent ammonium molybdate and 1.0 ml of 0.08 per cent stannous chloride. The color was permitted to develop without heating for 15 minutes and the samples were read at 700 $m\mu$ and compared to an inorganic phosphorus sample containing 1.0 $\mu g/ml$.

Paper chromatographic analysis.—The R_f values of the sample were checked against commercial preparations of the suspected nucleotides using filter paper chromatography. The solvents used were the sodium phosphate isoamyl alcohol system [1] and the phosphate-ammonium sulfate-propanol system [7].

RESULTS

The results reported here deal specifically with unfertilized eggs. The changes occurring in the acid-soluble nucleotides during development will be the subject of a future communication.

Fig. 1 shows a typical column separation of the acid-soluble nucleotides from unfertilized amphibian eggs. It is apparent that ten distinct ultraviolet-absorbing peaks are present in this fraction. Peaks Nos. 5 and 6 have not been

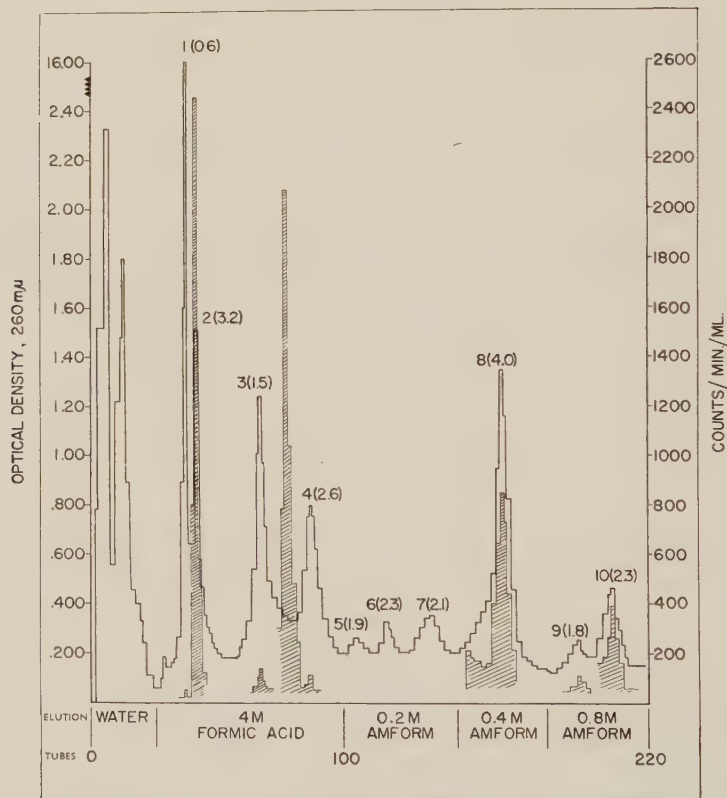


Fig. 1.—Column separation of acid-soluble nucleotides. Twelve hours after injection of P^{32} , samples of about two thousand eggs were taken and extracted.

Column: Dowex-1-formate, 1×15 cm column

Volumes: Tubes 1–30: 100 drops per tube

Tubes 31–50: 50 drops per tube

Tubes 51– : 100 drops per tube

Figures above each peak refer to the peak number; values in parentheses represent the 260/280 $m\mu$ ratios. Hatched lines indicate radioactivity present in 1.0 ml of fraction.

TABLE I. *Chromatographic data.*

Solvent A: 5 per cent aqueous Na_2HPO_4 , saturated with isoamyl alcohol (Descending).
 Solvent B: 0.1 *M* sodium phosphate buffer pH 6.8/ammonium sulfate/*n*-propanol 100/60/2 (Ascending).

Sample	Solvent	Temperature (°C)	R_f
Peak 1	A	20	0.89
cytosine-5'-phosphate			0.89
Peak 1	B	19	0.69
cytosine-5'-phosphate			0.69
Peak 2	A	19	0.72
adenosine-5'-phosphate			0.73
Peak 2	B	19	0.92
adenosine-5'-phosphate			0.92
Peak 3	A	19	0.74
guanosine-5'-phosphate			0.74
Peak 4	A	20	0.89
uridine-5'-phosphate			0.89
Peak 4	B	19	0.66
uridine-5'-phosphate			0.66
Peak 7	B	19	0.31
adenosine-5'-diphosphate			0.32
Peak 8	B	19	0.39
adenosine-5'-triphosphate			0.39
Peak 9	B	19	0.61
guanosine-5'-triphosphate			0.60
Peak 10	B	19	0.84
uridine-5'-triphosphate			0.83

identified because of insufficient quantities of material. This figure indicates that Peaks 2 and 8 are highly radioactive and that Peak 10 contains a significant amount of radioactivity. The radioactive peak between Peaks 3 and 4, which does not correspond to an ultraviolet-absorbing compound, is due to inorganic P^{32} . The ultraviolet absorbance of the water wash is presumably due to the presence of nucleosides and free bases.

This chromatographic picture is highly characteristic of amphibian egg material since ovarian eggs, unfertilized eggs, and developing embryos all yield similar results.

The 260/280 $m\mu$ ratios, the ultraviolet absorption spectra of the peak tubes, and the strength of the ammonium formate buffers necessary to elute these compounds from the column suggested that the 4 *N* HCOOH peaks

[1-4] were monophosphates of cytosine, adenosine, guanosine, and uridine respectively while the 0.4 *M* and 0.8 *M* ammonium formate peaks [8-10] were triphosphates of adenosine, guanosine, and uridine respectively. Consequently after the samples were dried *in vacuo*, paper chromatograms of the peaks were run with commercial preparations of the suspected nucleotides. The results of these determinations are shown in Table I. Fig. 2 is a representation of the chromatographic separation of the suspected triphosphate compounds.

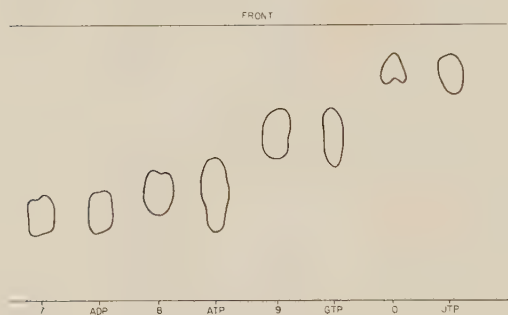


Fig. 2.—Chromatographic separation of the di- and triphosphates on filter paper. Commercial preparations of ADP, ATP, GTP, and UTP were run with the unknown peaks. Whatman #1, ascending for 16 hours, solvent was 0.1 sodium phosphate buffer, pH 6.8/ammonium sulfate/*n*-propanol 100/60/2.

TABLE II. *Chemical analysis.*

All analyses were made on compounds isolated from same clutch of eggs.

Peak No.	Max. in acid (m μ)	Min. in acid (m μ)	mM apparent pentose per mM base	mM labile phosphate per mM base	mM total phosphate per mM base	Base	Identification
1	278	240	0	0	1.4	cytosine	cytosine-5'-phosphate
2	258	230	1.1	0	0.9	adenine	adenosine-5'-phosphate
3	254	227	1.4	0	0.9	guanine	guanosine-5'-phosphate
4	260	230	0.2	0	1.8	uracil	uridine-5'-phosphate
7	258	231	1.0	1.3	1.9	adenine	adenosine-5'-diphosphate
8	258	245	0.9	2.2	2.7	adenine	adenosine-5'-triphosphate
9	254	243	0.9	1.6	3.7	guanine	guanosine-5'-triphosphate
10	260	247	0	1.5	2.4	uracil	uridine-5'-triphosphate

Although the chromatographic data had confirmed our suspicions it was felt that chemical analyses must be undertaken to definitely establish the identity of the compounds. Consequently pentose and phosphate molar ratios were determined on the peaks obtained from a single large sample. The results of these investigations are reported in Table II. The pentose is reported as "apparent" pentose because of the difficulty in quantitatively hydrolyzing the pyrimidine nucleotides. Using the diphenylamine reaction, it was found that there was no deoxyribose present in any of the compounds and it was therefore concluded that the nucleotides were of the ribose type.

DISCUSSION

Hultin [5], in a short note, recently reported the separation of the acid-soluble nucleotides from sea urchin eggs but the details of his analysis of the compounds were omitted. It was rather surprising that Hultin did not find guanosine triphosphate in his preparations.

This method of separation of the acid-soluble nucleotides permits not only the determination of the specific activities of the individual compounds but also allows the calculation of the concentrations of the nucleotides. Thus, during development of the amphibian embryo, it is possible to trace the transfer of phosphate within this fraction and to determine the concomitant changes in concentrations of the nucleotides. These experiments are currently in progress in this laboratory and it is already apparent that the AMP-ATP system is highly active in the amphibian eggs during development.

SUMMARY

1. Eight acid-soluble nucleotides from amphibian eggs have been separated chromatographically and chemically identified.
2. The nucleotides include the monophosphates of cytosine, adenosine, guanosine, and uridine; the diphosphate of adenosine; the triphosphates of adenosine, guanosine, and uridine.
3. Preliminary P^{32} incorporation data indicate that the AMP-ATP system is highly active in amphibian eggs.

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SYNCHRONIZED DIVISION OF GROWING CULTURES OF *ESCHERICHIA COLI*¹

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DURING the course of experiments on the effect of 2,4-dinitrophenol (DNP) on enzymes of *Escherichia coli*, it was noticed that addition of DNP to growing cultures did not affect the growth rate as measured by increase of turbidity, but that cell division was inhibited for a while and then the bacteria all divided at once, thus doubling the cell numbers within 10 minutes time [29]. Such an effect was also noted on addition of thyroxine and of several experimental nitrobenzimidazole compounds. For many years there have been reports in the literature of the accomplishment of synchronized cytoplasmic division in cultures of growing organisms brought about by various means, especially by changes in temperature [1, 2, 8, 15, 16, 17, 18, 27, 28, 31].

The possibility that such cultures which were synchronized to the extent that all cells divided at the same time might also be synchronized in other functions, such as the synthesis of cell components, especially the nucleic acids, has opened up fascinating vistas into the dynamics of cell growth and has also offered the hope that perhaps the effect of various drugs on growth might be localized. Hotchkiss [16] has used this method to show transformation by DNA during the period just before cell division of *Pneumococcus*.

Lark and Maaløe [18] have studied the relationship of the nucleic acid synthesis to cellular division in *Salmonella typhimurium* which were synchronized by heating from 25°C to 37°C and returned to 25°C.

Our preliminary experiments with division of fertilized *Chaetopterus* eggs at Woods Hole Marine Biological Laboratories showed that careful manipulation of temperature produced cultures in which of the 95 per cent of the eggs which were fertilized, 90 per cent divided together within an interval of about 5 minutes (see also Fry [13]). However, it was found that a "closed" system, such as the developing egg, was less easily manipulated than an

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“open” system, such as a bacterial culture. Therefore, *E. coli* B was chosen for further investigation, since it utilizes an exogenous food supply and can grow adequately in a medium in which all essential nutrients are inorganic except for a source of organic carbon.

In this paper are presented some investigations on the methods of producing synchronized division of *E. coli* by physiological means. The synchronizing effect of drugs will be handled in a later communication.

METHODS

Cultures.—The organisms used were wild type *E. coli* B, the original culture of which was obtained from S. S. Cohen. For indicated experiments a culture was obtained from the stock of J. Gots. Thus the inocula for these cultures were two different lines of *E. coli* B, which had been carried in two different laboratories for at least 10 years. Whether previous to that time the two lines had come from the same culture, is not known.

The cultures were carried on nutrient broth agar slants and were subcultured weekly in synthetic liquid medium “52”, containing ammonium sulfate, phosphates and MgSO_4 , CaCl_2 and FeSO_4 [12] to which glucose was added. The day preceding an experiment an inoculum of 0.001 to 0.10 of the weekly culture was added to 100 ml of “52” containing 2 mg/ml of glucose, and was incubated at 36°C with aeration overnight. Experimental cultures were grown in cylinders described by Cohen and Barner [9] which had sidearm tubes which fitted into the Klett–Summerson photometer. They were likewise equipped with a tube for adequate aeration. The medium was 200 ml of “52” containing glucose or other metabolites [12]. The inoculum from the overnight culture was taken directly, or centrifuged and washed, and then chilled to 5°C in an ice bath as required. Samples were taken as needed with sterile pipettes.

Turbidity was determined in the Klett–Summerson photometer, using either a 420 or a 660 filter, the latter when yellow compounds were added to the culture. Cell counts were obtained either by plate or by direct count microscopically in a Petroff–Hausser bacterial counter. Counts by the two methods agreed, so it was considered that direct count measured viable bacteria under conditions reported here and was, therefore, used in most experiments [15, 24]. Visual counting also allowed for the estimation of size and of filamentous forms.

At intervals during each experiment samples of the culture of suitable size for analysis (0.3 ml to 2.0 ml) were taken with sterile pipettes and transferred to tubes sitting in an ice-bath and containing 1/10 volume of N HCl . The tubes were centrifuged and the cells washed with saline or alcohol. The cells in one tube were analyzed for total Bial reactive material by the orcinol method of Miller and Miller [19]. Yeast RNA or ribose-5-phosphate were used as standards. The other sample was analyzed for total DNA by Dische’s [11] diphenylamine reaction, or the modification of that method by Burton [6], which includes addition of acetaldehyde to the reagent. With either reagent, the mixture was allowed to react overnight at room temperature, then boiled for 5 minutes, and read at 600 $\text{m}\mu$ by the Beckman spectrophotometer. Thymus DNA was used as a standard.

The bases, pyrimidines and purines (P & P), were estimated by measurement of absorption in the UV on the Beckman spectrophotometer. This was done on whole cells and extracted cells by suspension in 96 per cent glycerine with the same refractive index as the cells [3, 4, 10], or after solution in 1 N NaOH and neutralization to pH2. For calculation of nucleic acid, from the density at 260 m μ , the value for mixed nucleotides $E_p = 9.3$ was used for the extracts and for the dissolved cells, and the value $E_p = 6.2$ for naturally occurring nucleoprotein in calculation of nucleic acid in the clarified suspension [23]. The phosphorus content of the nucleic acids was considered to be 10 per cent [12].

In the medium supernatant to the cells, glucose was determined by the anthrone method of Mokrasch [21].

All results are expressed in terms of micrograms of nucleic acid per ml of culture for convenience of comparison, although, as will be discussed in the next paper, these values may not represent complete nucleic acids.

Results obtained by these methods on whole cells are within the range of those reported previously [7, 20, 22, 26]. The standard deviation of determinations on duplicate samples was from 2 to 7 per cent in all procedures. Any change of less than 10 per cent is, therefore, not regarded as significant except under certain conditions which will be discussed in the next paper.

RESULTS

Factors in the production of synchrony.—Preliminary attempts to synchronize division with cold treatment suggested that several variables affect the bacteria. Of the known factors which may possibly cause bacterial cells to divide synchronously, two will be discussed in this paper: first, production of synchrony of division by a growing culture by means of a change in temperature; and, second, by addition of glucose to a culture which has exhausted its substrate.

For temperature change we have used only chilling and not warming, and only one temperature change, from 36–37°C to 4.5–6°C, and back to 36–37°C. The time of chilling is important. By Fig. 1 it is evident that 15 minutes is too short. From 30 to 60 minutes brings the cells into a condition which arrests division for approximately 60 to 90 minutes, and then the cells may divide within the shortest period in which a count can be made, which is approximately 6 minutes.

For the second method, addition of glucose to a resting culture, the state of the bacteria is conditional. A resting culture is one that has ceased to grow because it has exhausted some essential nutrient. The length of time the culture has been in that state is important, that is, how long it has been

under conditions otherwise conducive to growth, i.e., aeration and temperature, after the nutrient was exhausted. The effect of these conditions is shown graphically in Fig. 2. The inocula for these two cultures had both exhausted their substrate. The inoculum for A culture had been incubated with aeration

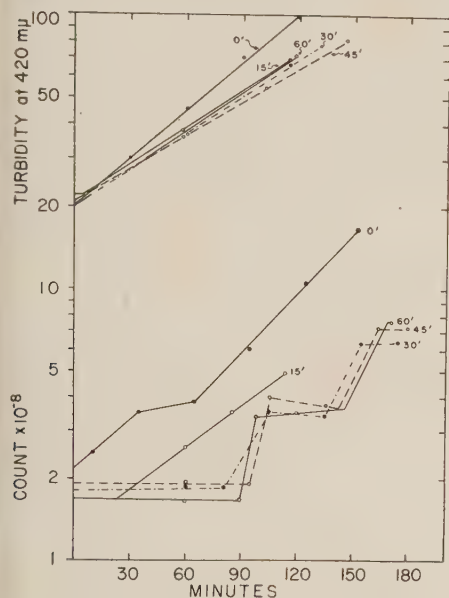


Fig. 1.

Fig. 1.—The inoculum was from a culture obtained by inoculating 0.01 ml of weekly culture into 100 ml of "52" medium plus 2 mg/ml of glucose incubated at 37°C with aeration for 17 hours. The inoculum was chilled, centrifuged and suspended in 40 ml of medium. This suspension was chilled at 4.5°C and after 15, 30, 45 and 60 minutes respectively 0.9 ml was inoculated into 20 ml of medium containing 2 mg/ml of glucose at 37°C. The 0' culture was directly inoculated from an overnight culture without chilling or centrifuging.

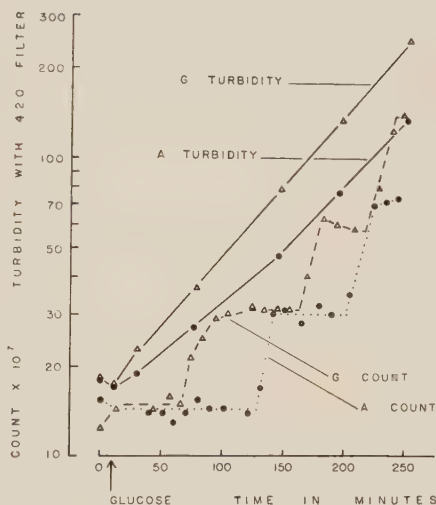


Fig. 2.

Fig. 2.—The inoculum for culture A was 1 ml of culture in medium originally containing 2 mg/ml of glucose which had been aerated at 37°C for 24 hours, turbidity at 420 $m\mu$ = 400. The inoculum for culture B was 1.4 ml of similar culture which had been aerated for 16 hours, turbidity = 300. Both inocula were chilled for 45 minutes at 5°C. Glucose 2 mg/ml, was added at 10 minutes. The higher turbidity of A culture was due to evaporation of water during the long aeration.

for 24 hours; the inoculum for G culture for 16 hours. The A inoculum had, therefore, reached the "stationary" phase approximately 8 hours before the G inoculum. The RNA/DNA ratio of G inoculum was 3.1, and of A, 2.2.

Fig. 3 brings together the results of this experiment and two other experiments to show that synchronization of division may be obtained by chilling a growing inoculum (A), or by using an inoculum which had exhausted its

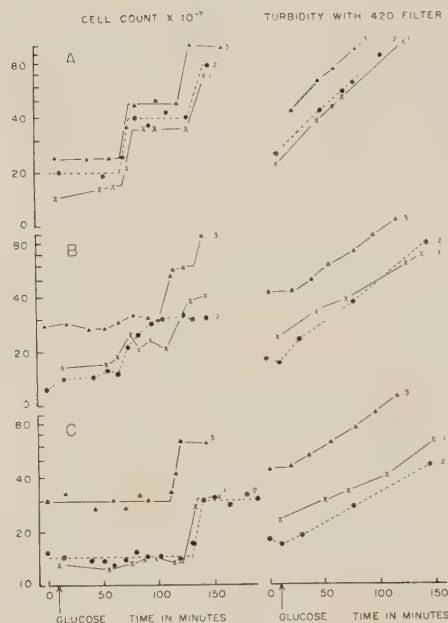


Fig. 3.

Fig. 3.—Inocula for cultures A1 and A2, B2 and C2 were the line from Dr. Cohen used in our experiments. Inocula for cultures A3, B1, B3, C1 and C3 were the line from Dr. Gots's laboratory. The inocula in Group A were cultures containing 2 mg/ml of glucose originally and still growing with a turbidity of approximately 200. The inocula in group B had depleted the glucose 1 to 3 hours previously. The turbidities were approximately 300. The inocula in group C had depleted the glucose at least 4 hours previously. The turbidities were from 330 to 400.

The inoculum for B1 and C1 was the same but, after being used for B1, it had stood in the refrigerator for 2 hours. The inoculum for A1 and A2 was also the same, and for A2 had also stood in the refrigerator for 2 hours; however, there was still glucose present in the inoculum medium so the cells were not depleted. The curves in Fig. 3 are from 3 different experiments.

All inocula were chilled 45 minutes at 6°C and put into warm media. For cultures A1 and A2, B1 and C1, the media contained glucose. The media for the other cultures did not contain glucose,

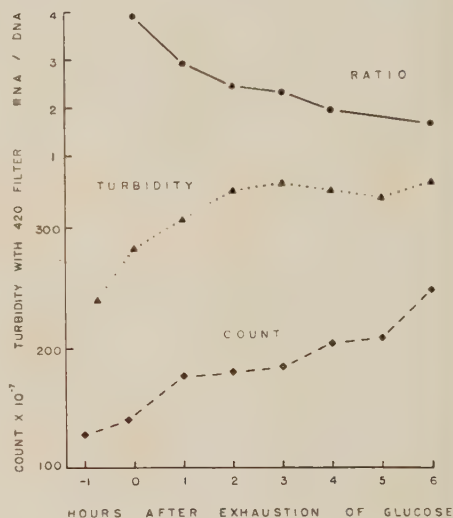
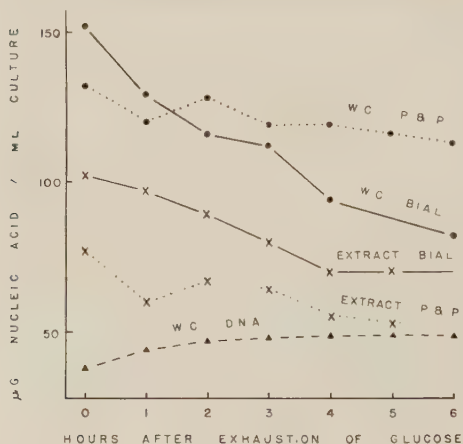


Fig. 4.

which was added 10 minutes after inoculation and aeration had been started.

Figs. 4 and 5.—100 ml of medium containing 2 mg/ml glucose were inoculated with 0.005 ml of weekly culture of *E. coli* B, and incubated with aeration for 24 hours, at which time the samples were taken hourly for analysis. W.C. DNA = diphenylamine reaction on whole cells; W.C. Bial = the orcinol reaction on whole cells; W.C. P & P = the purine and pyrimidine content calculated from the absorption at 260 mμ; Extract Bial = the orcinol reaction on the 5 per cent perchloric acid soluble fraction; and Extract P & P = the purine and pyrimidine content of the 5 per cent perchloric acid soluble fraction from the 260 mμ absorption. The ratio is RNA/DNA, calculated from the values on whole cells. The cells were very small at the last count.



substrate at least 4 hours previously (C), but not reproducibly by inocula obtained from cultures within the 4 hours after substrate exhaustion (B).

It may be seen that the cultures from growing inocula (A) divided 60 minutes after the glucose was added, while the cultures from inocula which had rested at least 4 hours (C) divided from 100 to 120 minutes after the addition of glucose. The intermediate cultures were not well synchronized in their division.

It would seem that during those 4 hours after exhaustion of glucose in the medium a slow change to the "depleted" state was taking place at a rate which was evidently not the same for all the cells of the culture, so that some cells divided at the time one would expect cultures from a growing inoculum to divide, and then 60 minutes later they all divided (B1 and B3), or division was spread over a longer interval, as in B2.

This phenomenon has been at least partially responsible for our previous difficulty in obtaining reproducible and complete synchrony and the one-half step patterns so often encountered in the earlier experiments [10, 30, 31]. For *E. coli* the inoculum for these earlier experiments was an overnight culture considered to be in the "resting" state, but how long it had been in this state was not known nor was it considered important.

Some of the changes that occur in the cells during the time after exhaustion of the substrate are shown in Figs. 4 and 5. Turbidity continued to increase 20 to 30 per cent and the count sometimes doubled. The DNA continued to increase 20 to 70 per cent in the first 3 hours, and then stabilized and remained constant. The Bial-reactive material decreased to 50 per cent of its maximum value. In consequence the RNA/DNA ratio fell (Fig. 4).

The depleted cells, when inoculated into fresh medium with a substrate for growth, proceeded to recover from depletion, or to prepare for growth [32]. The period of "lag", or before division, was then twice as long as that of cells which were already growing and had been chilled. During this preparation for growth, if the DNA cell was high initially, it increased 20 to 100 per cent, while the RNA, or pentose and P & P increased from 100 to 500 per cent.

The results of one such experiment are given in Table I, in which the strain of *E. coli* B was obtained from Dr. Gots. Two experiments with our strain are given in Table II. As may be seen, the bacteria in the culture grown from stock obtained from Dr. Gots were larger, that is, the turbidity per 10^8 cells was higher, and there were other differences, such as DNA/cell. However, as shown in Fig. 2, the division time patterns were similar.

As noted previously, the second way of producing synchronization was by

TABLE I

	Culture immediately after inoculation			Culture at beginning of or during division		
	Exp. I	II	III	Exp. I	II	III
Turbidity in inoculum	228	306	318			
Turbidity in culture	43	42	44	80	90	105
Count $\times 10^{-8}$	2.4	2.8	3.0	3.6 ^a	5.3 ^a	4.2 ^a
Time of beginning division				65	111	117
Time of sample				62	110	118
<i>γ/ml of culture</i>						
Whole cell						
Bial as RNA	19.5	13.0	8.1	44.0	48.0	57.0
Dische as DNA	4.0	5.0	4.6	7.1	10.0	11.2
P&P as NA	21.0	15.8	13.3	47.0	50.0	63.0
Extract						
Bial as RNA	15.0	12.0	8.0	19.2	33.2	37.0
P&P as NA	9.5	6.8	5.4	8.5	17.0	22.0
RNA/DNA	4.9	2.6	1.8	6.2	4.8	5.1
<i>$\gamma \times 10^{-8}$/cell</i>						
Bial as RNA	8.1	4.7	2.7	18.0	17.0	19.0
Dische as DNA	1.7	1.8	1.5	3.0	3.5	3.7
P&P as NA	8.7	5.7	4.4	20.0	18.0	21.0
Extract						
Bial as RNA	6.2	4.3	2.7	8.0	12.0	12.0
P&P as RNA	4.0	2.4	1.8	3.5	6.1	7.3
Turbidity/ 10^8 cells	18.0	15.0	14.7	33.0	32.0	35.0

^a Time of counts.—These were first counts after division started. These counts were not used for calculation.

addition of an essential nutrient to a resting culture. In the experiments reported here the essential nutrient for *E. coli* B was the carbon source, glucose. Certain mutant strains of *E. coli* have specific nutrient requirements which can also be added to resting cultures to initiate synchronized growth. *E. coli* 113-3, derived from W strain, requires Vitamin B₁₂, and can be synchronized by addition of either Vitamin B₁₂ or methionine. The purine-requiring mutant *E. coli* B96 will start synchronized growth by addition of adenine or guanine. Barner and Cohen [5] have used the thymine-requiring mutant *E. coli* 15T-, and found that cultures in medium complete except for thymine divided

TABLE II

	Experiment No. IV		Experiment No. V		
	Initial	Before division	Initial	Before division	
Turbidity	43	65	35	66	
Count $\times 10^{-8}$	4.0	4.5	2.7	5.5 ^a	
Time of division		64		45	to 52
Time of sample		57		48	51
<i>γ/ml of culture</i>					
Whole cell					
Bial as RNA	10.4	24	12.0	26.0	41.7
Dische as DNA	7.9	9.7	7.3	12.4	20.6
P&P as NA	15.9	28	20.0	31.3	38.7
Extract					
Bial as RNA	5.0	9.3	12.2	17.3	20.0
P&P as NA	3.2	5.3	12.3	17.8	17.2
RNA/DNA	1.32	2.5	1.6	2.1	2.0
<i>γna/10⁸ cells</i>					
Bial as RNA	2.6	6.0	4.4	9.6	15.5
Dische as DNA	2.0	2.4	2.7	4.6	7.6
P&P as NA	4.0	7.0	7.4	11.6	14.3
Extract					
Bial as RNA	1.2	2.3	4.5	6.4	7.4
P&P as NA	0.8	1.3	4.5	6.6	6.4
Turbidity/10 ⁸ cells	11	16	13	25	

^a Count at end of division.

synchronously after thymine was added if the period of exposure to the incomplete medium was not so prolonged that death of the cells had occurred.

A study of the sequence of biochemical syntheses in synchronized cultures will follow.

SUMMARY

Some of the conditions which lead to the state of synchronous division in cultures of *E. coli* B have been investigated. Two reproducible methods for the achievement of this state are the chilling and rewarming of a growing culture, and the addition of glucose to a culture which has been incubated

for at least 4 hours after depletion of glucose from the medium. During the period of depletion the RNA/DNA ratio dropped. After addition of glucose this ratio rose.

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THE EFFECT OF STARVATION ON EPITHELIAL RENEWAL IN THE RAT DUODENUM

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THE existence of a constant renewal of the epithelial lining of the intestine is now well established. The suggestion was made by Bizzozero [1] that the mitotic activity of the epithelium in the crypts supplies cells which crowd up to cover the base of the villi. This has been amplified and confirmed experimentally by Friedman [6] who demonstrated that goblet cells swollen by X-rays migrate from the crypts far up onto the villi. Leblond and Stevens [10] postulated that the whole epithelium is constantly renewed by cell formation in the crypts and migration of the cells up the sides of the villi to the extreme tips, where cells are lost by extrusion into the lumen (Fig. 1). This thesis has been proven by experiments which traced the movement of cells labelled with radioactivity [11, 16]. In normal animals the number of cells covering the surface remains approximately constant because the rate of cell formation equals that of cell loss. A complete renewal of the epithelium is accomplished in 1-3 days in rats and mice, as judged by the results of mitotic counts [10, 17] and experiments using radioactive labels on the cells [11, 16].

In the present work, the effect of a period of starvation on epithelial renewal in the duodenum of rats has been investigated. Restriction of food intake has previously been shown to have a depressing effect on mitotic activity in several tissues, including the intestinal crypts [3-5, 9, 12, 17]. However, the action of starvation on other aspects of renewal has not been consistently examined. Our studies include the measurement of (1) the numbers of epithelial cells per crypt and villus, (2) the mitotic activity of the crypts, and (3) the percentage of villi showing cells in extrusion, which it was hoped would provide an indication of the rate of cell loss.

MATERIALS AND METHODS

In three similar experiments using young adult rats of Sherman stock, control animals were fed Purina Fox Chow and water *ad libitum*, whereas the starved rats were only given water. All animals were sacrificed 5 days later. In Experiment 1,



Fig. 1.—Normal duodenal mucosa. The crypts (C) are lined by epithelium with many mitotic cells (M indicates metaphases). The cells move up onto the sides of the villi (V) and glide up to the tips, where they are extruded. Small round white spaces in the epithelium are mucus of goblet cells. Haematoxylin and eosin. $\times 135$.

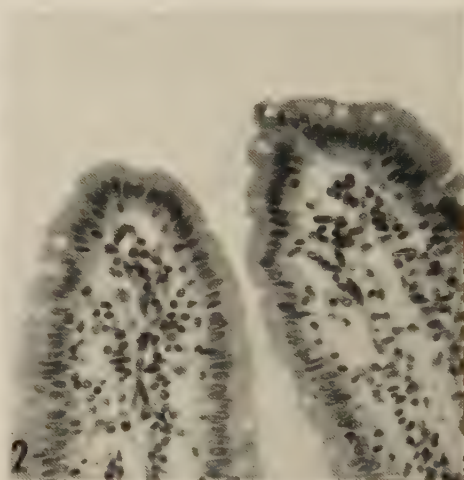


Fig. 2.—Tips of two duodenal villi. The nuclei and cell boundaries of the epithelial layer covering the villus on the left form a constant, unbroken line typifying the absence of an extrusion zone. In contrast to this, the tip of the epithelial layer covering the villus on the right is marked by a protruding of the cell membranes and the lack of continuity in the position of the nuclei. This is characteristic of an extrusion zone.

four control and six starved female rats were used and sacrificed between 10:00 a.m. and 12:00 noon. Experiment 2 consisted of six control and seven starved female rats which were sacrificed between 3:30 and 4:00 p.m. In Experiment 3, four control and four starved male rats were sacrificed between 4:00 and 4:30 p.m. The animals were sacrificed with chloroform. Portions of the proximal duodenum were immediately removed, cut open longitudinally, flattened gently on cardboard and placed in Zenker-Formol fixative. Paraffin sections for the first and third experiments were cut longitudinally at 6μ and those of the second experiment at 4μ . All sections were stained with Harris' haematoxylin and eosin.

The number of interphase and mitotic epithelial cells in histological sections was estimated by counting their nuclei. The nuclei of chief, goblet and Paneth cells, but not those of migrating leucocytes were included in the counts. Those nuclei were counted which were at least half included in the section, as judged by their sectional diameter and staining intensity. The counts were made as follows:

The crypt and villus cell population size.—Areas which showed a continuous epithelial layer from the tip of the villus to the base of the crypt were selected to insure

that the full length of both structures was seen. The number of nuclei lining one side of such a crypt and villus was then recorded. Although it was difficult to find many such perfectly sectioned areas, the number of cells in 8–20 crypts and villi were measured per animal.

The number of cells in mitosis per crypt.—Thirty crypts, cut throughout their length, were examined per animal, and the number of nuclei in each stage of mitosis from early prophase to late telophase was recorded per crypt. A nucleus was judged to be in prophase when the chromatin was collected into flakes and accumulated along the nuclear membrane. As mitosis progresses the nuclei move up in the cell so that the metaphases, anaphases and early telophases lie well above the level of the interphase nuclei, whereas the later stages sink back towards the basement membrane. In late telophase the pairs of daughter nuclei tend to be slightly smaller and more oval than interphase nuclei, and their chromatin is arranged in thin strands.

The percentage of villi with extrusion zones.—100–200 villus tips were examined per animal and classified as to whether they did or did not have extrusion zones. Only those villi were examined in which the section passes through the tip, as shown by the presence of a continuous single layer of nuclei in the epithelium (Fig. 2, left). Such a villus was said to have an extrusion zone when one or more cells were seen to be detached from the basement membrane and seemingly squeezed up between neighbouring cells and protruding above the normal epithelial surface (Fig. 2, right).

RESULTS

The rats starved for five days seemed in good general health despite an average loss of about 18 per cent of their initial body weight (Table I). At autopsy, the intestines of the starved animals were generally empty except for the presence of some material in the terminal ileum and a few small faecal pellets in the colon. In control animals varying amounts of food material was found in the stomach and small intestine, but the ileum invariably contained considerable quantities of digested matter and the colon, at least a few normal faecal pellets. The mucosa of the starved animals appeared normal under both gross and microscopic examination. No consistent difference from that of control fed animals was seen as far as the mucosal thickness, shape of the villi, epithelial continuity, or size and cytology of the epithelial cells was concerned. Particularly, the degenerative changes, epithelial splits and loss of structure of the villi in intestines of mice starved almost to death as described by Diller and Blauch [5] and Sun [15] were not observed in our work.

The results of the counts of interphase and mitotic nuclei and of extrusion zones are shown in Table I. Statistical analysis of the results showed that following starvation there is a significant reduction in the number of epithelial cells lining the crypt walls. In female rats sacrificed in the morning there was

TABLE I. *Effect of five days starvation on body weight and epithelial renewal of rat duodenum.*

Group	G body weight Mean $\pm$ s.e.		No. cells per side of crypt Mean $\pm$ s.e. ^a	No. cells per side of villus Mean $\pm$ s.e. ^a	No. mitoses per crypt Mean $\pm$ s.e. ^a	% villi with extrusion zones Mean $\pm$ s.e. ^a
	Initial	Final				
<i>Exp. No. 1</i>						
Control	240 $\pm$ 9.5	240 $\pm$ 10.7	71.7 $\pm$ 6.8	150.6 $\pm$ 13.2	7.3 $\pm$ 0.6	59.4 $\pm$ 2.7
Starved	250.7 $\pm$ 9.3	205 $\pm$ 8.5	58.6 $\pm$ 2.8 $P < 0.001^b$	102.8 $\pm$ 2.6 $P = 0.001$ -0.01 ^b	6.4 $\pm$ 0.6 $P = 0.001$ -0.01 ^b	58.9 $\pm$ 2.2 $P = 0.8-0.9^b$
<i>Exp. No. 2</i>						
Control	183.3 $\pm$ 7.5	188 $\pm$ 6.8	56.3 $\pm$ 1.8	114.0 $\pm$ 8.8	7.1 $\pm$ 0.8	57.9 $\pm$ 4.8
Starved	195 $\pm$ 5.4	155.7 $\pm$ 4.7	48.2 $\pm$ 1.6 $P < 0.001^b$	97.0 $\pm$ 4.7 $P < 0.001^b$	5.4 $\pm$ 0.3 $P = 0.001$ -0.01 ^b	36.9 $\pm$ 1.9 $P = 0.001$ -0.01 ^b
<i>Exp. No. 3</i>						
Control	135 $\pm$ 5.8	176 $\pm$ 7.4			6.6 $\pm$ 0.6	62.4 $\pm$ 2.7
Starved	147 $\pm$ 3.2	125 $\pm$ 2.6			5.1 $\pm$ 0.2 $P < 0.001^b$	65.2 $\pm$ 2.9 $P = 0.5-0.6^b$

^a Standard error as used in *t*-test.^b *P* value derived from an analysis of variance.

a mean reduction of about 18.2 per cent, and in the afternoon, the mean value in fasted rats was about 14.4 per cent less than that of the fed rats.

A decreased number of epithelial cells lining one side of the villi was also found following starvation. The average loss in female rats sacrificed in the morning and afternoon was 31.7 and 14.9 per cent respectively, as compared with the values in control animals.

The difference between the number of mitoses per crypt in fed and fasted animals was not significant when the results were analyzed by the *t* test. This finding has been reported [2, 8]. However, reexamination of the results by an analysis of variance showed that despite the wide variation between animals within each group, starvation caused a significant depression in the mitotic activity of the crypt epithelium. Thus, in starved female animals sacrificed in the morning (when the control rats have just finished a period of eating), mitosis was reduced by 12.3 per cent on the average (Experiment 1), and

by 24.0 per cent (Experiment 2) in rats sacrificed in the afternoon (when the stomachs of control rats are generally empty) and to the extent of 22.7 per cent in male rats sacrificed in the afternoon (Experiment 3).

The values of the percentage of villi bearing extrusion zones did not show consistent changes in the three experiments. A significant reduction occurred in the villi of starved female rats sacrificed in the afternoon, but no changes were found in male animals sacrificed at this time, nor in female rats sacrificed in the morning.

DISCUSSION

The results of these three experiments show that the number of cells lining one side of the crypts and villi is moderately reduced in starvation (Table I). Since the crypts and villi are sectioned randomly, we may conclude that the number of cells covering the whole of these structures is decreased; there is a smaller cell population in the intestinal epithelium. How may we explain this finding? In the intestinal epithelium of normally fed animals the cell population remains constant because the rate of cell formation equals the rate of cell loss; the epithelium is in a steady state. A decrease in the population size, such as brought about by starvation, might be due to an increase in the rate of cell loss or a decrease in cell production, or both.

The possibility of an increased loss of cells has received little attention, perhaps because it is difficult to measure cell loss directly. As the intestinal epithelium of our starved rats presented a normal appearance, presumably any cell loss must have occurred by the normal route, that is by extrusion of cells from the tips of the villi. Although the factors causing detachment of cells from this zone have not been identified, the muscular contractions occurring in the intestinal wall and the villi themselves during digestion may play a role in loosening the cells [8]. Decreased functional activity during starvation would decrease this muscular activity and thus remove one normal stimulus to cell extrusion. It was in an attempt to obtain some direct evidence on this matter that the numbers of villi bearing extrusion zones were counted. The finding that the percentage values were either unchanged (Experiments 1 and 3) or lower (Experiment 2) in starved animals suggests, but unfortunately does not prove, that the same or a smaller number of cells respectively was being extruded at the instant of fixation in control and starved animals.

The second most likely cause of a reduction in cell population size is a decrease in the mitotic rate. Our finding of a significant decrease of about 12–24 per cent in the number of mitoses per crypt in starved rats is in agree-

ment with this hypothesis. A similar but more severe effect of starvation was observed by Sun [15] and also by Diller and Blauch [5] who found a decrease of about 80 per cent in the mitotic activity of small intestinal epithelium in mice which had been starved almost to death.

A decreased rate of cell production may lead indirectly to a decreased rate of cell loss. This concept rests on the supposition that the loss of cells from the villus tip is, to some extent, a consequence of the pressure exerted by the cells crowded up from the regions of cell formation. Reduced mitotic activity in the crypts of starved animals would then reduce the pressure of cell migration up the villi and the cells at the tip would be less squeezed from all sides. Some evidence that pressure is not the only cause of cell extrusion was derived from the observations of Grad and Stevens [7] on the intestinal epithelium of rats injected with radiophosphorus. Although mitotic activity in the crypts of these animals was destroyed by radiation, the epithelial cell population of the crypt and villi decreased, presumably due to continuing cell extrusion without the normal means of replacement.

Thus, starvation definitely causes a reduction in the rate of cell formation and may also reduce cell loss. Even if no changes in cell loss were to occur, the fall in mitotic activity in Experiments 1 and 2 (12.4 and 24 per cent respectively) would be sufficient to account for the decrease in the number of cells lining crypts and villi (27.5 and 14.7 per cent respectively), since the 5 day experimental period is more than twice the time required for complete renewal of the epithelium. It does not, of course, follow that these changes are consistent throughout the period of starvation. Perhaps the most likely explanation of what occurs is that for a time the rate of cell formation is less than that of cell loss, but eventually a new balance is struck so that a reduced cell population is maintained with a slower rate of renewal. This sequence of events probably occurs in chronically underfed animals and may even pertain in starved animals until they become moribund.

SUMMARY

Young adult rats of both sexes were deprived of food for five days, but allowed free access to water. These animals lost about 18 per cent of their body weight but remained in good condition.

Histological examination revealed a normal appearance of the duodenal mucosa of starved rats as compared with those of control fed animals.

The results of two separate experiments showed a significant decrease in the number of cells lining one side of the crypts (18.2 per cent, 14.4 per cent)

and of the villi (31.7 per cent, 14.9 per cent) in histological sections of starved rats.

The mean number of dividing cells per crypt in three groups of starved rats was decreased 12.3–24.0 per cent below that in control groups.

This reduction in the size of the epithelial cell population in starved rats may be explained by an imbalance in the steady state of renewal of the epithelium which exists in fed animals. Presumably the reduced rate of cell formation is exceeded by the rate of cell loss, at least for a time, so that the cell population becomes smaller.

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ANALYSE AUTORADIOGRAPHIQUE DE L'INCORPORATION DE LA PHÉNYLALANINE-2-¹⁴C DANS LES NOYAUX ISOLÉS

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LES travaux de Mirsky et de son école [1, 3, 4] ont montré que des noyaux isolés de leur cytoplasme sont capables d'incorporer un acide aminé : ces auteurs ont constaté, en effet, que l'alanine-¹⁴C peut marquer de façon appréciable les protéines des noyaux de thymocytes de veau. Ces noyaux, isolés dans le saccharose, ne présentent pas de perte ou d'échange de protéines solubles et conservent intact leur appareil enzymatique. Le taux d'incorporation de l'acide aminé est beaucoup plus élevé dans les noyaux de thymus que dans les noyaux de foie de rat ou de rein de porc — isolés par le même procédé. Par contre, les noyaux de thymocytes, isolés en milieu non aqueux, selon Behrens, n'incorporent pas d'acides aminés dans leurs protéines.

Allfrey [1, 3] a précisé que l'incorporation de l'acide aminé dans les protéines nucléaires du thymus nécessite une source d'énergie, faute de quoi elle est diminuée de 25 à 50 %. Enfin, l'intégrité de l'acide désoxyribonucléique (ADN) paraît être une condition indispensable à la synthèse des protéines nucléaires. Plus exactement, un prétraitement par la désoxyribonucléase (DNase) inhibe l'incorporation de 40 à 85 %, pour un traitement de 15 et 75 minutes respectivement. L'addition d'ADN de thymus (à l'inverse de l'ADN de germes de blé) restaure presque complètement l'incorporation dans les protéines. Le ribonucléase (RNase) n'a pas d'effet inhibiteur sur l'incorporation.

Diverses critiques ont été faites à Mirsky et à ses collègues au sujet de la pureté de leurs préparations nucléaires, une légère contamination cytoplasmique pouvant modifier considérablement les conditions de l'incubation *in vitro*. C'est ainsi que certains auteurs [5, 6] ont tenté de montrer, par une méthode de réfractométrie, que cette contamination est beaucoup plus importante que celle observée par l'école de Mirsky. En contrôlant leurs préparations par microscopie électronique et par des analyses enzymatiques (cytochrome oxidase) et des dosage de l'ARN, Mirsky et ses collègues [2] ont estimé à 29 % le nombre de thymocytes intacts et à 10 % au total, le nombre des noyaux contaminés.

Il nous a paru intéressant de reprendre ce problème par la méthode autoradiographique, dans le but de vérifier si la présence éventuelle de cytoplasme et si l'état physiologique du noyau peuvent influencer cette incorporation; si celle-ci a lieu, par exemple, à un moment particulier de l'interphase, correspondant à une synthèse d'ADN.

Cette méthode offre, on le sait, l'avantage d'apporter des données sur les noyaux individuels.

MATÉRIEL ET MÉTHODES

Au cours d'une série de 7 expériences, les noyaux de thymocytes de veau ont été isolés en suivant exactement les conditions décrites par Allfrey [1]. A la fin de l'isolement, effectué à 2°, en 45 minutes, la suspension compte environ 25 mg de noyaux/ml.

Les conditions de l'incubation, en aérobiose à 37°, sont analogues à celles décrites par Mirsky *et al.* [3]. Chaque lot est constitué de 0,5 ml de suspension nucléaire, 0,2 ml de glucose · NaCl · MgCl₂ et 0,25 ml de saccharose · phosphate à pH 7,3.

La phénylalanine-2-¹⁴C a été administrée à 3 concentrations différentes : 1, 2 et 6 μ C respectivement par ml de suspension.

Dans le but d'étudier l'effet de la DNase et de la RNase sur l'incorporation de l'acide aminé, les 2 enzymes ont été utilisés chacun à la concentration de 500 γ /ml de suspension. Dans certaines expériences les noyaux subissent un traitement préliminaire de 30' à 37° en aérobiose : dans ce cas, les contrôles étaient mis à incuber dans les mêmes conditions, pendant 30 minutes, sans l'enzyme; dans d'autres expériences, les noyaux étaient traités simultanément par l'enzyme et la phénylalanine-2-¹⁴C, pendant toute la durée de l'expérience, c'est-à-dire pendant 3 heures.

Un essai de réactivation par de l'ADN de thymus de veau a également été tenté. Dans ce cas, l'action enzymatique de la DNase a été arrêtée après 30 minutes par l'addition de citrate et l'ADN a été ajouté à raison de 5 mg/ml.

Deux expériences préliminaires ont eu pour but de rechercher si l'irradiation U.V. (2200 ergs/mm²)¹ ou X (950 r)² exerce une inhibition sur l'incorporation de l'acide aminé marqué dans les noyaux isolés.

Enfin, une expérience a été effectuée en l'absence de radioisotope, afin de contrôler l'effet du traitement *in vitro* sur la colorabilité des noyaux. A la fin de cette même expérience, nous avons dosé par la méthode de Schneider [14] l'ADN, tant dans les noyaux contrôles que dans ceux qui avaient été traités par la DNase.

Nous avons comparé 3 procédés de fixation différents : l'alcool-acide acétique (3:1), le liquide de Zenker et l'acide trichloracétique à la concentration de 10 %, d'une part sur les noyaux en frottis sur les lames histologiques, d'autre part sur les noyaux rassemblés en culot par une centrifugation douce. Le but de ces essais était de voir si une éventuelle pellicule cytoplasmique ne se détache pas du noyau au moment de l'étalement des frottis.

¹ Lampe Mineralight donnant 90 % de l'énergie à 253,7 m μ et calibrée à l'aide d'un photomètre U.V. de Latarjet [9]. Toutes les manipulations consécutives à l'irradiation U.V. sont effectuées à l'obscurité.

² Délivrées en environ une minute à partir d'un tube Machatt à fenêtre de Béryllium sous 50 kV, 30 mA; filtre de 1 mm Al.

Au cours de l'incubation en présence de phénylalanine-2- ^{14}C , des prises du matériel ont été faites après 10', 20', 40', 50', 2 heures et 3 heures.

Quelques gouttes de la suspension de noyaux marqués ont été étalées sur des lames gélatinées, séchées rapidement et fixées pendant 10' dans un mélange d'alcool et d'acide acétique (3:1). Après un lavage dans l'alcool à 70°, les lames ont séjourné pendant 15' dans une solution de phénylalanine non radioactive.

Les préparations ont été soumises alors à l'autoradiographie (Nuclear track emulsion, Ilford, G5, in gel form) selon le procédé décrit ailleurs [7].

La durée de l'exposition des autoradiographies a généralement été de 4 jours. Cependant, dans le but de vérifier si le rapport des noyaux marqués aux noyaux non marqués demeure le même en prolongeant cette exposition, nous avons laissé certaines préparations pendant 8, 12 et 20 jours en chambre noire avant de les développer.

Après le développement photographique et un lavage poussé, les noyaux ont été colorés par la méthode de Unna. Des mesures histophotométriques ont été effectuées sur des noyaux colorés au vert de méthyle pour voir s'il existe une relation entre la concentration en ADN et la radioactivité du noyau à l'aide d'un histophotomètre de Lison [11]¹.

RÉSULTATS

Après 3 heures de traitement *in vitro*, les noyaux présentent une allure parfaitement normale. Les microphotos (fig. 1 et 2) montrent l'aspect de ces noyaux après qu'ils ont été recouverts de l'émulsion photographique où se sont inscrites les traces des électrons émis par la phénylalanine-2- ^{14}C .

Les résultats consignés dans les tableaux I, II et III correspondent aux 3 expériences ayant trait à l'action des nucléases et à celle des irradiations sur l'incorporation de l'acide aminé marqué.

Le tableau IV montre les relations entre les noyaux marqués et les noyaux non marqués, en fonction de la durée de l'exposition des autoradiographies. Le tableau V présente les résultats de l'incorporation en fonction des différentes concentrations en radioisotope du milieu.

Toutes ces valeurs correspondent aux différentes classes de noyaux : non marqués, marqués par 1, 2, 3 et éventuellement 4 traces.

Le tableau VI présente les résultats du dosage de l'ADN dans les différentes fractions des noyaux témoins et traités pendant 3 heures par la DNase.

¹ Nous remercions vivement M. le Professeur Pasteels qui a aimablement mis son appareil à notre disposition.

Fig. 1.—Autoradiographie de noyaux isolés de thymocytes ayant incorporé de la phénylalanine-2- ^{14}C dans leurs protéines. On observe les traces des électrons émis par le ^{14}C , dans l'épaisseur de l'émulsion nucléaire.

Fig. 2.—Autoradiographie de noyaux isolés de thymocytes ayant incorporé de la phénylalanine-2- ^{14}C dans leurs protéines. On remarque que le noyau à gauche, en bas, est recouvert d'une pellicule cytoplasmique et n'est pas radioactif.

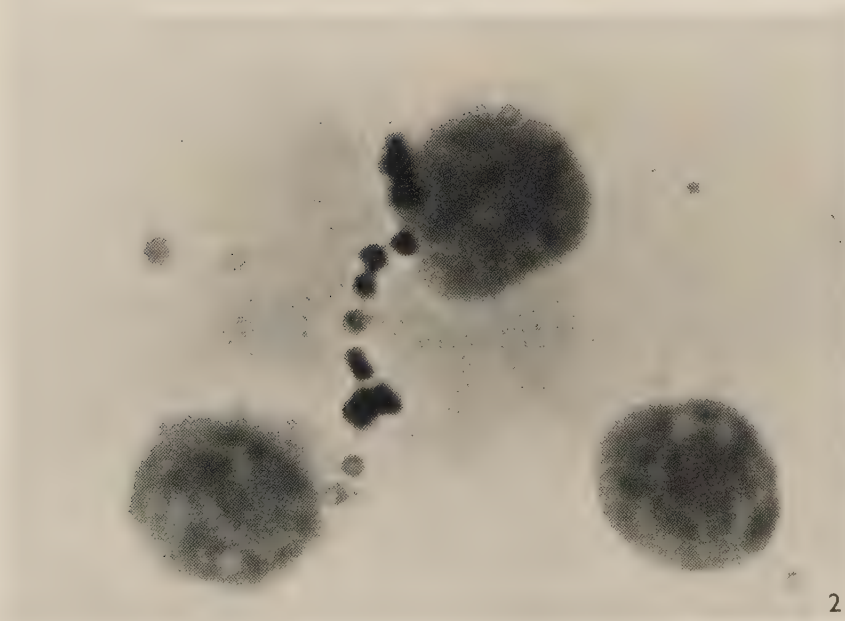
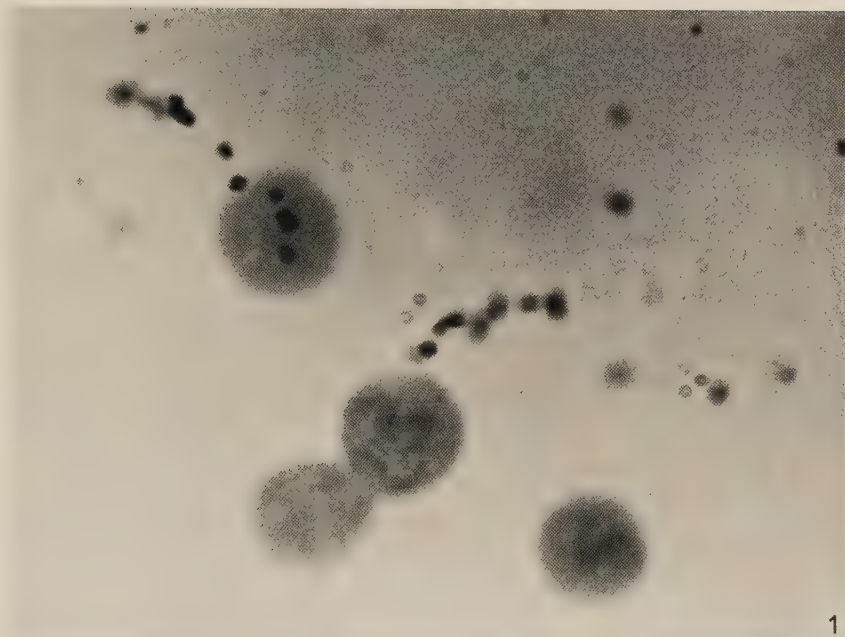


TABLEAU I

Durée du traitement <i>in vitro</i> (min.)		Noyaux non marqués	Noyaux marqués par:		
			1 trace	2 traces	3 traces
5	Témoins	962	38	—	—
	RNase	952	48	—	—
	DNase	970	30	—	—
	U.V.	968	31	1	—
10	Témoins	887	107	6	—
	RNase	925	74	1	—
	DNase	948	25	—	—
	V.V.	972	28	1	—
20	Témoins	884	110	4	—
	RNase	883	113	—	—
	DNase	958	42	—	—
	U.V.	968	32	—	—
40	Témoins	831	150	19	—
	RNase	844	148	8	—
	DNase	945	55	—	—
	U.V.	927	70	3	—
60	Témoins	633	287	77	3
	RNase	670	254	75	1
	DNase	880	120	—	—
	U.V.	784	190	26	—

Radioactivité : 1 $\mu\text{C}/\text{ml}$ de suspension de noyaux.

L'incorporation de la phénylalanine-2- ^{14}C dans les noyaux isolés est linéaire au cours du temps. Le traitement par la DNase (500 γ/mC) et l'irradiation U.V. (2200 erg/mm^2) inhibent l'incorporation de 50 à 70 %.

DISCUSSION

Ces résultats sont comparables à ceux obtenus par Mirsky et ses collaborateurs. Nous avons pu confirmer que :

(1) les noyaux isolés sont capables d'incorporer un acide aminé (la phénylalanine) *in vitro*;

(2) la DNase (500 γ/ml) inhibe de 50 à 70 % l'incorporation, qui est restaurée par l'addition d'ADN de thymus de veau (5 mg/ml);

TABLEAU II

Durée du traitement <i>in vitro</i> (min.)		Noyaux non marqués	Noyaux marqués par:		
			1 trace	2 traces	3 traces
10	Témoins	955	45	—	—
	R.X.	956	44	—	—
	DNase	952	48	—	—
	DNase + ADN	947	53	1	—
20	Témoins	924	76	—	—
	R.X.	928	72	—	—
	DNase	950	50	—	—
	DNase + ADN	920	80	—	—
40	Témoins	888	110	2	—
	R.X.	918	81	1	—
	DNase	953	47	—	—
	DNase + ADN	898	102	—	—
60	Témoins	762	224	12	2
	R.X.	908	92	—	—
	DNase	898	102	—	—
	DNase + ADN	820	180	—	—

Radioactivité : 2 μ C/ml.

(3) un prétraitement par la RNase (500 γ /ml pendant 30') n'a qu'un effet négligeable sur l'incorporation. Il serait utile de vérifier, cependant, que l'enzyme pénètre réellement dans le noyau. Mirsky a fait remarquer que, dans les conditions de travail que nous avons adoptées, 30 % seulement de l'ARN nucléaire sont détruits.

Nous avons observé en outre que :

(4) l'irradiation X ou U.V. inhibe d'environ 50 à 70 % l'incorporation; dans le cas des rayons X, celle-ci paraît être partiellement restaurée par l'addition d'ADN;

(5) la radioactivité des noyaux n'est pas proportionnelle à la concentration en radioisotope.

L'autoradiographie a permis de préciser encore que:

(6) les noyaux isolés ne se marquent que dans une proportion relativement faible : dans les meilleures conditions, après 3 heures de contact avec

TABLEAU III

Durée du traitement <i>in vitro</i>		Noyaux non marqués	Noyaux marqués par :		
			1 trace	2 traces	3 traces
20 min.	Témoins	899	92	9	—
	DNase	942	58	—	—
	R.X.	872	134	—	—
	R.X. + ADN	910	90	—	—
40 min.	Témoins	847	130	22	1
	DNase	925	75	—	—
	R.X.	922	75	3	—
	R.X. + ADN	878	112	—	—
60 min.	Témoins	734	186	72	8
	DNase	—	—	—	—
	R?X.?	954	46	—	—
	R.X. + ADN	896	102	2	—
2 hrs	Témoins	672	226	84	8
	DNase	960	40	—	—
	R.X.	884	104	12	—
	R.X. + ADN	840	154	6	—
3 hrs	Témoins	622	248	109	21
	DNase	905	95	—	—
	R.X.	876	112	14	—
	R.X. + ADN	806	190	4	—

Radioactivité : 2 μ C/ml.

Le traitement par la DNase (500 γ /ml) et l'irradiation X (950 r) inhibent l'incorporation de l'acide aminé marqué, d'environ 50 %. Dans les 2 cas, l'addition d'ADN de thymus (5 mg/ml) restaure presque totalement la capacité d'incorporation.

le radioisotope, environ 60 % des noyaux sont marqués (même après 20 jours d'exposition en chambre noire); on observe, par exemple, que pour une durée triple ou même quintuple d'exposition des autoradiographies, un pourcentage double seulement de noyaux se marquent. Cependant, quand la densité des traces est très élevée; il semble qu'on fasse une erreur d'observation par défaut.

(7) le contrôle de la pureté des préparations nucléaires a été effectué, comme nous l'avons dit, par la coloration d'Unna. Nos observations, qui

TABLEAU IV

Durée du traitement <i>in vitro</i>	Durée de l'exposition (jours)	Radioactivité des différentes classes de noyaux marqués en fonction de la durée de l'exposition des autoradiographies						
		Noyaux non marqués	1 trace	2 traces	3 traces	4 traces	Total des traces	% des noyaux marqués
20 min.	4	884	110	4	—	—	118	12
	12	757	161	56	26	—	351	25
40 min.	4	831	150	19	—	—	188	17
	12	661	183	133	22	1	519	34
60 min.	4	734	186	72	8	—	354	27
	12	515	225	205	60	5	835	49
3 hrs	4	622	248	109	21	—	529	38
	12	435	275	190	80	20	975	57

Le nombre de noyaux qui se marquent au cours de l'exposition des autoradiographies n'est pas proportionnel à la durée de cette exposition.

ont porté sur 1000 noyaux, révèlent une proportion de 3% de cellules intactes et 8% de noyaux recouverts d'une mince pellicule cytoplasmique [2]¹.

De toutes façons, nous n'avons tenu compte, dans nos mesures, que des noyaux réellement propres. Nous avons d'ailleurs constaté que la présence de cytoplasme ne modifie pas sensiblement l'incorporation dans les noyaux contaminés, du moins dans le cas des thymocytes.

Après 35' de contact avec la DNase, les noyaux ne se colorent plus au vert de méthyle. Toutefois un dosage des noyaux traités par l'enzyme pendant 3 heures ne montre qu'une diminution de 15% de la teneur en ADN; on retrouve celui-ci dans le milieu d'incubation.

La colorabilité des noyaux est partiellement restaurée par l'addition d'ADN de thymus.

Les mesures histophotométriques, effectuées sur les noyaux colorés au vert de méthyle, semblent montrer qu'il existe un petit nombre de noyaux

¹ Nous avons également fait quelques contrôles à l'aide d'un microscope interférentiel Baker, après avoir suspendu les cellules dans une solution concentrée de Serum albumine. Le nombre des cellules intactes est du même ordre de grandeur que celui trouvé après coloration ce qui correspond assez bien aux valeurs trouvées par Allfrey et Mirsky [2].

TABLEAU V

Durée du traitement <i>in vitro</i>	Radioac- tivité du milieu d'in- cubation (μ C)	Noyaux non marqués	Noyaux marqués par:			Total des traces
			1 trace	2 traces	3 traces	
75 min.	2	652	260	88	—	436
	6	679	231	90	—	411
135 min.	2	641	255	104	—	463
	6	545	327	123	5	588
3 hrs	2	555	262	155	28	651
	6	495	321	159	25	714

La radioactivité des noyaux n'est pas proportionnelle à la concentration en radioisotope du milieu.

TABLEAU VI

	Témoins	Témoins 3 heures	DNase 3 heures
Milieu extérieur	0	460	700
Extrait acidosoluble des noyaux	450	450	450
ADN	2170	1920	1650
Total	2620	2830	2800

γ d'ADN.

Après 3 heures de traitement *in vitro* en présence de DNase (500 γ /ml), 15 % de l'ADN passent dans le milieu.

qui sont plus radioactifs que les autres, qui sont plus volumineux et qui fixent davantage le vert de Méthyle. Mais le nombre des mesures effectuées sur des noyaux fortement marqués (qui sont peu nombreux) est trop faible pour qu'on puisse tirer des conclusions.

Un calcul statistique préliminaire nous avait conduits à penser que l'incorporation ne se fait pas selon une distribution de Poisson, laissant supposer que la population de noyaux que nous avons isolée n'est pas homogène, du moins en ce qui concerne le phénomène de l'incorporation. Nous avons

déjà vu, en effet, que même en prolongeant l'exposition des autoradiographies jusqu'à 20 jours, une forte proportion de noyaux demeure non marquée. La classe des noyaux marqués par plus de 2 traces semble, elle aussi, être en excès sur la distribution prévue. Ces faits qui, à priori, peuvent donner à penser que l'incorporation se produit à un moment défini de l'interphase, doivent cependant être interprétés avec réserve : récemment, Levi [10] qui a étudié quantitativement les causes d'erreurs de la méthode autoradiographique que nous avons utilisée, a observé des distributions de radioactivité du même type, pour des objets inorganiques, en principe homogènes. Il pourrait donc ne s'agir là que d'un artefact.

Des expériences actuellement en cours dans notre laboratoire [13] sur des noyaux isolés de foie de rat, indiquent que les noyaux des thymocytes ne sont probablement pas les seuls à être capables d'incorporer des acides aminés *in vitro*.

Un mécanisme d'anabolisme protéique peut donc exister dans le noyau, indépendamment du cytoplasme. Ce mécanisme n'est pas nécessairement le seul qui intervienne dans la synthèse des protéines nucléaires, puisque, dans le cas des noyaux de foie, l'addition d'une fraction cytoplasmique peut stimuler ce métabolisme nucléaire [13].

Une fois de plus, les relations entre acides nucléiques et protéines sont évidentes, à l'échelle, cette fois, du noyau isolé. Il reste à préciser les rôles respectifs de chacun des acides nucléiques qui pourront être établis de façon plus sûre par des dosages que par l'autoradiographie. Un point, en effet, reste à élucider : c'est le rôle de la DNase. Comme on l'a vu, des dosages biochimiques ont montré que l'enzyme ne détruit que 15 % de l'ADN du noyau isolé, à une concentration et pour une durée de traitement qui inhibe de 75 % l'incorporation.

On peut se demander dès lors, si la DNase n'agirait pas indirectement sur l'ARN, affectant ainsi le métabolisme protéique. En effet, certains auteurs [8] ont attiré l'attention sur l'existence d'une action compétitive entre l'ADN et l'ARN pour la RNase et, dans une plus faible mesure, entre l'ARN et l'ADN pour la DNase. Cette compétition serait due à la formation de complexes entre l'enzyme et l'un ou l'autre des deux acides nucléiques. Une telle interprétation permettrait peut-être d'expliquer plus facilement les restaurations, observées par Mirsky et Allfrey à l'aide de divers polynucléotides : ceux-ci déplaceraient la désoxyribonucléase du complexe, biologiquement inactif.

RÉSUMÉ

L'autoradiographie a permis de vérifier que la contamination cytoplasmique ne semble pas modifier d'une manière importante l'incorporation de la phénylalanine radioactive dans les protéines de noyaux isolés de thymus.

Certains résultats sont en faveur de l'idée que l'incorporation de l'acide aminé est concomitante à la synthèse d'ADN dans le noyau.

SUMMARY

It has been possible, with radioautography, to verify that cytoplasmic contamination does not essentially modify the incorporation of radioactive phenylalanine into the proteins of isolated thymus nuclei. Certain results indicate that the incorporation of the amino acid may be simultaneous to nuclear DNA synthesis.

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THE DEOXYRIBONUCLEIC ACID CONTENT OF GUINEA-PIG ADRENAL NUCLEI AFTER ADMINISTRATION OF CORTICOTROPIN

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It is generally accepted that endogenous or exogenous corticotropin (ACTH) decreases the amount of lipid and ascorbic acid in the cells of the adrenal cortex of laboratory animals [19] and of man [23]. Recently, however, it has been appreciated that ACTH also produces marked changes in the nucleic acids of the adrenal gland [24, 25]. The increase in ribonucleic acid (RNA) in the adrenal is associated with the appearance of the "compact" type of cell. This "compact" cell is usually seen in the *zona reticularis* and is in marked contrast to the lipid-laden "clear" cell usually seen in the *zona fasciculata*.

In estimating the total amount of RNA, and other substances, in a tissue it is sometimes convenient to relate the measurement to some parameter more constant than the weight of the tissue. Fiala, Sproul and Fiala [10], in studying the nucleic acid and protein content of the adrenal of the rat after treatment with ACTH, presented their results as values per cell. They estimated the number of cells present in a given gland by measuring the absolute deoxyribonucleic acid (DNA) content of the gland and then dividing this value by the amount of DNA found per cell of the untreated adrenal of that species. Most investigators believe that the mean DNA content of the resting diploid somatic cell is constant for any one species since the amount of DNA is dependent on the number of chromosomes present in the cell [12, 13, 28]. Though this is generally accepted, some authors claim to have found variations from these mean values for the same tissues in the same species. Where these variations in mean values have been detected by cytophotometric methods [14, 15, 16, 22], they may be explained by errors in technique or by variations in the mitotic activity of the tissue in question [1, 7, 8, 17, 26, 27, 31, 32]. In tissues where DNA synthesis is occurring actively, the mean DNA value tends to be elevated since the DNA content of the rapidly growing cell doubles itself during interphase prior to cell division [29].

We have found [18] that ACTH causes an increase in the total amount of

DNA per gland in the rat and in the guinea-pig, and also considerable enlargement of the nuclei of the cells in the *zona fasciculata* and *zona reticularis* of some species [3]. There is also, under conditions of stress, increased mitotic activity [20]. It therefore appeared unwise to assume, as Fiala *et al.* [10] have done, that the mean DNA content of these nuclei remained constant during the period of treatment with ACTH. We therefore decided, before proceeding with other experiments, to measure by chemical methods the mean DNA content of adrenal nuclei isolated from animals which had received ACTH, and also to determine the scatter about this mean by cytophotometry. As a standard of reference it was decided to employ nuclei isolated from the kidney, since the kidney has been shown to be composed almost entirely of diploid cells [11], and since this organ was presumed to be unaffected by ACTH administration. As experimental animal the guinea-pig was chosen since it possesses a relatively large adrenal and responds readily to exogenous ACTH. A preliminary account of part of this work has already appeared [4].

MATERIALS AND METHODS

Animals.—Female guinea-pigs of body weights ranging from 350–500 g were used. The animals were divided into groups of 6 with approximately the same average body weight, and all groups were maintained and handled in a similar manner.

ACTH administration.—Each of the 6 animals in each test group received an intramuscular injection of 5 mg of ACTHar gel (Armour Laboratories) morning and evening. The injections were continued throughout the experimental period and all animals in each group were killed on the morning of the day following the last injection. Control animals were those which received no ACTH.

Isolation of nuclei. The animals were killed by sudden stunning followed by bleeding from the neck vessels. Both adrenals and the left kidney were rapidly removed, dissected free of adhering tissue and weighed. Nuclei were isolated from the pooled adrenals and from the pooled kidneys of the 6 animals in each group. The nuclei from some of the control groups of animals and from the 1, 5, and 7 day test groups were isolated by the citric acid method described by Smellie, Humphrey, Kay and Davidson [21]. Nuclei from the remaining control groups and from the 3, 10, 14, and 21 day test groups were isolated by the sucrose-calcium chloride method of Allfrey, Mirsky and Osawa [2].

Chemical analysis.—Samples of the nuclear suspensions were pipetted off for chemical analysis and the number of nuclei per unit volume in the suspension was measured using a haemocytometer.

The samples of nuclei were treated with 0.5 vol. 30 per cent (w/v) trichloroacetic acid (TCA), spun in a refrigerated centrifuge and the precipitate washed twice with 10 per cent TCA (w/v). Supernatants were discarded and the residual material extracted successively with acetone, ethanol, ethanol chloroform 3:1 (twice), ethanol-ether 3:1, and ether. The dry residue was extracted twice with 0.5 N per-

chloric acid at 70°C for 20 minutes. The extracts were combined and their content of DNA determined by the method of Ceriotti [5].

Cytophotometric analysis.—Nuclei from the kidney of a control guinea-pig were smeared on one end of all 3" / 1" microscope slides. One specimen of nuclei from the control or test series was smeared on the other end of the slides. These smears were dried in air and fixed in acetic acid ethanol (1:3) for 18 hours. All specimens were hydrolysed in *N* hydrochloric acid at 60°C for 20 minutes, washed in distilled water and placed in leucofuchsin [6] for 45 minutes. The slides were then washed in 3 changes of acid metabisulphite solution [9], dehydrated, cleared, and mounted in DePeX (Gurr). The nuclei were photographed under conditions of Köhler illumination with mercury green light (546 m μ), using a 1.3 n.a. apochromatic oil-immersion condenser and a 1.3 n.a. apochromatic oil-immersion objective. The exposures were arranged so that the density of the background and of the object on the negatives fell within the range of the straight part of the gamma (time-density) curve of the Kodak microfilm film which was used. Thus there is a direct relationship between the density of the object image on the film and the density of the object. Approximately 40 nuclei of the control and of the test specimens were photographed together with a rotating step wedge sector on the same strip of film. The negatives were scanned in a direct recording microdensitometer [30] so that 3 separate traces across the image of the object were obtained. The total absorption of the object and the mean value for the DNA content in arbitrary units were then calculated from the average of these extinction values and the projected area of the image. This method greatly reduces the errors attributable to sampling by spot measurements [7]. Since the object is an isolated nucleus which has been smeared on a slide and dried in air, the error due to out-of-focus effects should be small.

RESULTS

The results of the chemical analyses are shown in Table I.

The mean values for the DNA content in arbitrary units obtained by the cytophotometric technique are given in Table II. The fact that these latter units are of the same order as those values given in picograms ($g \times 10^{-12}$) in Table I is purely coincidental. Histograms of some of the cytophotometric results on sucrose-calcium chloride nuclei are shown in Fig. 1. Nuclei from the citric acid preparations were also examined but were considered unsuitable because of the inhomogeneous dispersion of the material in them. Figs. 1*a* and 1*b* show a comparison of a control kidney and a test kidney. Figs. 1*c* and 1*d* show the values obtained from a control kidney and a control adrenal. Figs. 1*e* and 1*f* compare a control kidney and a test adrenal. It will be seen that an occasional nucleus in the adrenal has approximately the tetraploid amount of DNA. The number of such nuclei is very small; we have found only 11 out of 509 nuclei examined, and the proportion in the control and test adrenals is approximately the same. The other histograms

TABLE I. Mean values obtained by chemical analysis for the absolute DNA content per nucleus of the kidney and adrenal gland of guinea-pigs after treatment with ACTH for varying periods.

Days of ACTH administration	DNA per nucleus (pg)			
	Kidney		Adrenal	
	Citric acid isolation	Sucrose CaCl ₂ isolation	Citric acid isolation	Sucrose CaCl ₂ isolation
0	6.5	5.9	7.2	7.7
1	6.7		7.6	
3		6.4		7.6
5	6.4		7.2	
7	7.0		7.6	
10		6.2		7.4
14		6.5		7.4
21		6.1		7.7

TABLE II. Mean values obtained by cytophotometry for the relative DNA content in arbitrary units per nucleus of the kidney and adrenal gland of guinea-pigs after treatment with ACTH for varying periods. Isolation of nuclei in sucrose-calcium chloride.

Days of ACTH administration	DNA content in arbitrary units			
	Test kidney	Corresponding control kidney	Test adrenal	Corresponding control kidney
3	7.2	7.4	7.5	7.9
10	8.0	7.2	7.7	7.7
14	7.6	7.8	7.8	7.0
21	7.9	7.7	8.3	8.9

for the remaining specimens show a distribution similar to that illustrated by Fig. 1. Histograms of the projected areas of the nuclei from the test adrenals are shown in Fig. 2. It is evident that the areas of the adrenal nuclei increase during treatment with ACTH. Despite this increase in projected area the amount of DNA per nucleus remains constant since the extinction decreases. The relationship between the concentration of DNA in the nucleus and the weight of the adrenal gland is shown for one group of experiments in Fig. 3. It is seen that the weight of the pooled glands from each group of 6

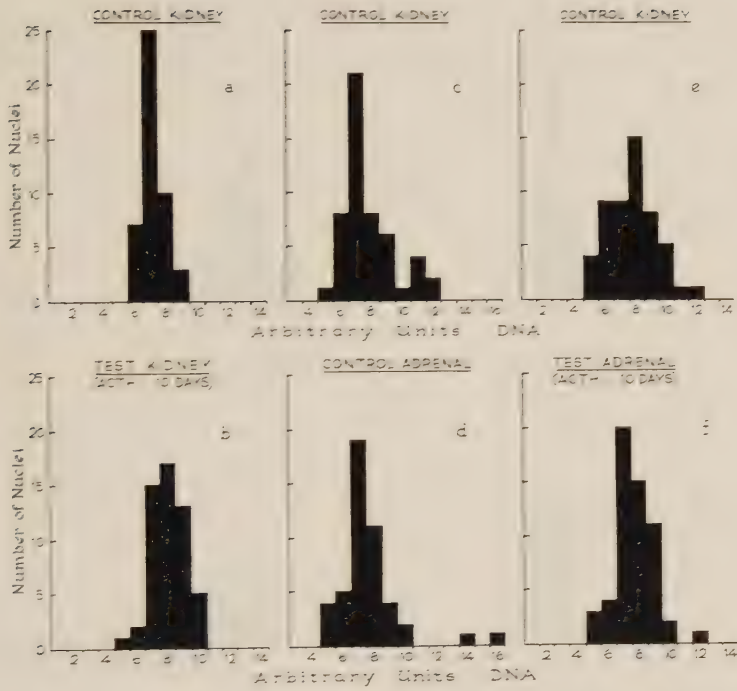


Fig. 1.—Histograms showing the patterns of distribution of DNA content of control and test samples of nuclei: (a) and (b) from control and 10-day test kidneys; (c) and (d) from control kidney and control adrenal; (e) and (f) from control kidney and 10-day test adrenal.

animals increases with continuing administration of ACTH. At the same time there is a decrease in the nuclear concentration of DNA expressed as the average extinction divided by the average projected area per nucleus.

DISCUSSION

The results show that although the administration of ACTH to guinea-pigs causes an increase in the size of nuclei isolated from the adrenal, it does not affect the mean DNA content of these nuclei. The total DNA remains constant despite the drop in concentration of DNA in these enlarged nuclei. If our results are generally applicable to the adrenals of all animals it is thus permissible, as Fiala *et al.* [10] have done, to estimate the number of cells present in the adrenal by measuring the total DNA content of the adrenal and dividing it by the mean DNA value per cell for that gland.

From the results of the chemical analyses it will be seen that the adrenal

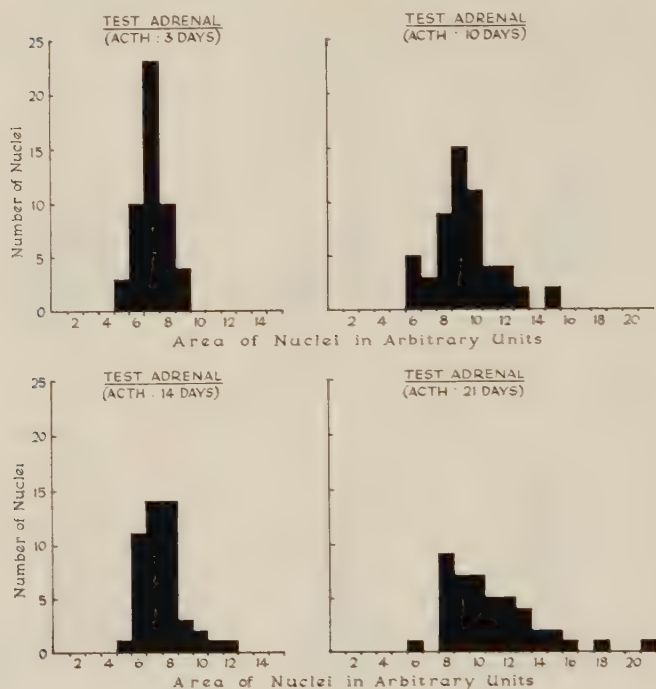


Fig. 2.—Histograms showing the patterns of distribution of the areas of adrenal nuclei after different periods of administration of ACTH.

nuclei appear to have a higher DNA content than the kidney nuclei. We have found that, with the guinea-pig adrenal gland, homogenization beyond 15 to 20 seconds leads to a considerable reduction in the number of nuclei in the suspension. In the isolating media we have used, the adrenal nuclei appear to be more fragile than the kidney nuclei. Thus one possible explanation of the higher values which we have obtained throughout for adrenal nuclei may be that these nuclei were slightly contaminated with nuclear fragments which had not been removed during the differential centrifugation. This would have the effect of raising slightly the value for the mean DNA content per nucleus. The mean value for kidney nuclei which we have obtained is close to the mean value for liver nuclei in guinea-pigs reported by Vendrely [28].

The cytophotometric results show no appreciable difference between the mean values for the adrenal nuclei and those for the corresponding control kidney nuclei. These results would not, of course, be influenced by any slight contamination by nuclear fragments.

The isolated nuclei come from all parts of the cortex and from the medulla of the gland. Further investigation [3] suggests that those nuclei in the guinea-pig adrenal which are enlarged lie in the *zona fasciculata* and the *zona reticularis*.

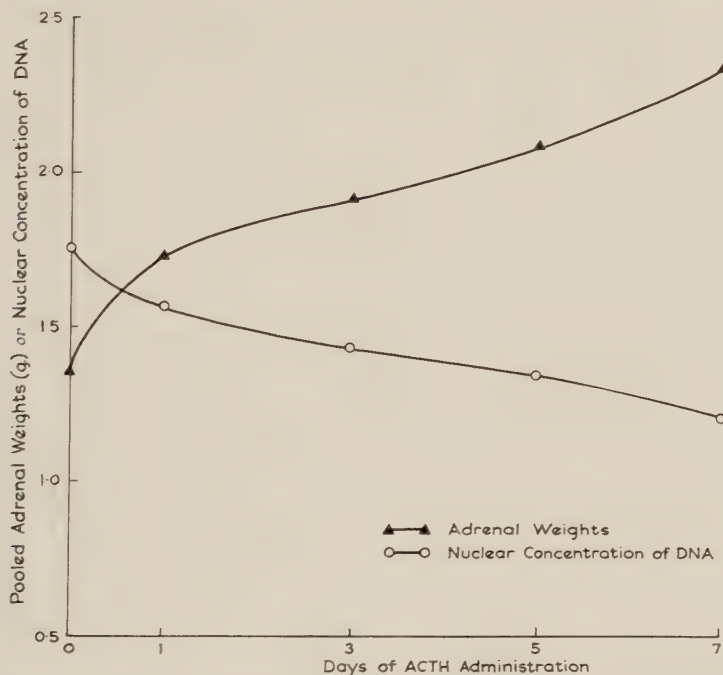


Fig. 3.—The effect of ACTH administration on the weight of the pooled adrenal glands per group of 6 guinea-pigs and on the mean nuclear concentration of DNA expressed as

$$\frac{\text{average extinction value}}{\text{average projected area}}$$

SUMMARY

1. The effect of corticotropin (ACTH) on the amount of deoxyribonucleic acid (DNA) per nucleus in the guinea-pig adrenal has been studied by chemical and cytophotometric methods.

2. In spite of the fact that ACTH causes considerable enlargement of the nuclei, the mean DNA content per nucleus is unaffected.

3. The evidence suggests that, in the guinea-pig, the adrenal nuclei are almost entirely diploid, and remain so even when the gland is stimulated by giving exogenous ACTH.

4. It is thus justifiable to determine the number of cells in the adrenal by dividing the total amount of DNA in the gland by the average amount of DNA per nucleus.

We should like to record our appreciation of the interest shown by Professor J. N. Davidson and Dr. H. S. D. Garven in this work, and to thank Miss E. A. McHutchon and Miss E. F. Dick for skilled technical assistance. One of us (A. J. H.) received a grant from the Secretary of State for Scotland, on the recommendation of the Advisory Committee on Medical Research, for the purchase of the recording microdensitometer.

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BRIEF NOTES

NUCLEIC ACID PATTERNS IN THE PANCREAS OF HYPOPHYSECTOMIZED RAT, AFTER ADMINISTRATION OF GROWTH HORMONE AND OF THYROXINE¹

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It has been shown that the atrophic pancreas [8] of hypophysectomized rat increases in weight after administration of growth hormone [3]. The administration of thyroid powder has the same effect [2]. However, the actions of the growth hormone (GH) and of the thyroid hormone seem to be different, since only the latter is able to raise the pancreas amylase activity depressed by previous hypophysectomy [5, 2]. In an attempt to elucidate the effect of these two hormones in the growth process, the desoxyribonucleic acid (DNA) and pentosenucleic acid (PNA) contents of the pancreas of the hypophysectomized rats were studied after administration of GH and thyroxine, separately or in combination.

Our experiments have been performed in a 2 · 2 factorial design. Sixteen Wistar female rats were hypophysectomized one month before the beginning of the injections. The animals were randomized and divided in four groups (blocks) of four animal each. In each block the animals were randomized; the first animal received subcutaneously 0.5 ml of saline, the second 0.5 ml of a 0.1 per cent Wilson's growth hormone (Lot 95567) solution, the third 0.5 ml of 0.05 per cent Roche's DL-thyroxine solution, and the fourth was injected with 0.5 ml of the growth hormone plus 0.5 ml of thyroxine solution. The injections were repeated daily over a period of six days. On the seventh day the animals were killed by ether. The pancreas was removed, weighed and immediately homogenized in ice-cold distilled water. PNA and DNA were assayed by the Schneider, Schmidt and Thannhauser procedure [7, 6]. The mean values of the findings are recorded in Table I. The results of the analysis of variance made on the original data are summarized in Table II. To save space, Table II gives only the mean squares and the F values.

It may be seen from the highly significant values of the F variance ratio that both thyroxine ($F = 14.8$) and growth hormone ($F = 17.7$) significantly raise the weight of the atrophic pancreas. The action of the combined hormones is purely additive as indicated by the non-significant F values for interaction. While the total DNA per organ is not affected by thyroxine treatment ($F = 1$), the concentration of this

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TABLE I. *Experimental results (means and standard error).*

Treatment.

	Hypo- physectomy	Hypo- physectomy plus thyroxine	Hypo- physectomy plus thyroxine and growth hormone	Hypo- physectomy plus growth hormone
Initial body weight in g	108 ± 7.3 ^a	104 ± 5.3	115 ± 6.9	109 ± 5.7
Final body weight in g	106 ± 6.6	87 ± 5.7	105 ± 5.1	120 ± 8.0
Pancreas weight in mg	193 ± 13.8 ^b	243 ± 13.8	305 ± 13.8	248 ± 13.8
Total DNAP per pancreas in µg	102.5 ± 7.36	108.5 ± 7.36	139.5 ± 7.36	134.5 ± 7.36
Total PNAP per pancreas in µg	295.0 ± 17.94	439.7 ± 17.94	578.0 ± 17.94	412.0 ± 17.94
DNAP µg/100 mg wet weight	52.7 ± 1.64	48.2 ± 1.64	46.0 ± 1.64	54.5 ± 1.64
PNAP µg/100 mg wet weight	152.2 ± 8.50	194.2 ± 8.50	189.7 ± 8.50	165.2 ± 8.50

^a Standard error of the mean calculated separately for each plot.^b Standard error per plot estimated from the residual error of the analysis of variance of the 2 × 2 factorial experiment.TABLE II. *Analysis of variance performed on the data of the 2 × 2 factorial experiment.*

	Action of thyrox		Action of GH		Interaction thyrox × GH		Between blocks		Experimen- tal error MS
	MS	F	MS	F	MS	F	MS	F	
Pancreas weight	11,342	14.8**	13,572	17.7**	49	1°	908	1.2°	768
Total DNAP	121	1°	3,969	18.3**	1	1°	356	1.64°	217
Total PNAP	96,565	26.9**	65,152	18.1**	451	1°	4483	1.2°	3589
DNAP µg/100 mg wet weight	169	15.9**	0.25	1°	16	1.5°	13	1.2°	10.6
PNAP µg/100 mg wet weight	4,422	15.1**	72	1°	306	1.0°	27	1°	294

MS = Mean square; F = Variance ratio; ** indicates significant values at the 1 per cent level; * significant at the 5 per cent level; ° non significant at the 5 per cent level.

substance expressed in $\mu\text{g}/100$ mg wet weight is significantly lower ($F = 15.9$). A similar result is observed in the animals which received the combined hormone treatment. On the other hand both the total PNA content and the PNA concentration raise significantly after administration of thyroxine ($F = 26.9$ and $F = 15.1$). Growth hormone, on the contrary, affects essentially the total content of DNA and PNA per organ; it does not alter significantly the concentration of nucleic acids on a wet weight basis.

Cytochemical examination of gallocyanine-stained sections from pancreas of thyroxine injected animals showed an increase in the basophilic material of the cytoplasm. This is also an indication of an increase of PNA in the cell. Growth hormone administration does not produce alteration in basophilic material stained by the same method. The pancreases of the GH-injected animals are cytologically quite similar to those of the hypophysectomized rats; they present less basophilic material and fewer zymogen granules than those of the pair-fed controls. These results indicate that growth hormone apparently affects the process of cellular multiplication, without modifying appreciably the cytoplasmic growth, since the total DNA increases in the whole organ without a corresponding increase in the concentration of PNA.

Thyroxine administered to hypophysectomized animal lowers the DNA concentration in the gland and raises the PNA concentration. The first effect would indicate a cytoplasmic growth without cellular multiplication, and the second one a raise in cytoplasmic nucleic acid content.

Mandel *et al.* [4] observed that thyroxine administration to normal rats raises the pancreas PNA and DNA contents. We are inclined to think that this increase in total DNA in the normal animal is due to an indirect action of thyroxine through the hypophysis, probably by liberating growth hormone [1].

Data on the amylase activity of the treated animals as well as the results observed on pair-fed controls will be published elsewhere.

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SWELLING OF THE NUCLEI OF *ASTERIAS* EGGS¹

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IN a previous paper [1] it was reported that the swelling of nuclei of *Asterias* eggs in 4.8 *M* glycerol varies with pH, being minimal at pH 7.4. In that investigation, a phosphate-citrate (McIlvaine) buffer was used in the pH range 7.0–7.4 and a veronal buffer in the pH range 7.5–8.0. It therefore seemed possible that the observed changes in nuclear swelling might be attributable not to the differences in pH but to specific effects of the ions or molecules of the buffer's used. The investigations have therefore been extended in an effort to resolve this problem.

Asterias eggs were treated as previously described by immersion in solutions of 4.8 *M* glycerol containing trishydroxy methylaminomethane (tris), glycine ethyl ester, and veronal buffers. The pH range covered was 7.1–8.0 the intervals being approximately 0.1 pH unit. With the tris and veronal buffers minima in the mean nuclear diameters ($n = 20$ in all cases) were found at pH 7.4, and with the glycine ethyl ester the minimum was at pH 7.35. In Table I the mean nuclear diameters at the pH intervals where minima were found and those of the adjacent intervals are given together with the standard deviations of the means. In all but two instances the differences between the means of adjacent intervals are on the border line of being statistically significant. Thus 6 of the total of 12 differences have a probability of occurring by chance of 0.06 or less, and 10 of the 12 differences have a probability of occurring by chance of 0.13 or less. The data therefore suggest that there is an effect of pH on nuclear swelling, a minimum being found at pH 7.35–7.42, but they do not conclusively prove the existence of this effect.

Although with these buffers, the minimum was found at approximately the same pH as that previously observed with combination of MacIlvaine and veronal buffers, the magnitude of the effect is very much less, suggesting the operation of other factors than pH in producing this phenomenon. To obtain further information on the nature of the other factor or factors comparison was made of the effects of MacIlvaine and of phosphate buffers on nuclear swelling in glycerol with results as shown in Tables II and III. In the MacIlvaine-buffered solutions minimum swelling was observed at pH 7.4 as in previous experiments. However, in the phosphate-buffered solutions there was no nuclear swelling at pH greater than 7.0. The appearance of the cells and nuclei in the latter solutions was markedly different from the appearance in MacIlvaine-buffered solutions or those buffered with tris, glycine ethyl ester, or veronal. The cytoplasm was very granular while the nucleus was hyaline except for the nucleolus. Nuclei extruded from their cells at pH 7.1 or higher were irregular in outline suggesting a "soft" and partially collapsed condition. There was thus a striking difference in the behavior of the buffers containing citrate as well as phosphate as compared with the buffers containing only phosphate. The results indicated

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TABLE I. Summary of differences in mean nuclear diameters at pH 7.2-7.5 and their standard errors. All in 4.8 M glycerol.

Buffer	Expt.	pH	m	σm	d	σd	$d/\sigma d$	
Tris (0.065 M)	1 ^a	7.20	15.4	0.77				
		7.42	12.9	1.00	2.5	1.26	2.0	
		7.48	13.2	0.83				
		7.60	15.7	0.96	2.5	1.27	2.0	
	2	7.22	18.1	1.08	1.5	1.61	0.9	
		7.42	16.6	1.18				
		7.48	18.7	0.92	2.1	1.42	1.5	
	Glycine ethyl ester (0.065 M)	1	7.25	18.4	0.54	2.0	0.99	2.0
			7.35	16.3	0.83			
7.42			19.5	0.46	3.2	0.95	3.4	
2		7.25	19.3	0.35	0.9	0.55	1.6	
		7.35	18.4	0.45				
		7.42	20.5	0.39	2.1	0.59	3.4	
Veronal (0.325 M)	1	7.31	20.5	0.45	1.6	0.85	1.9	
		7.39	18.9	0.73				
		7.52	20.5	0.60	1.6	0.94	1.7	
	2	7.31	19.9	0.99	2.1	1.59	1.3	
		7.39	17.8	1.25				
		7.52	20.3	0.92	2.5	1.55	1.6	

All measurements are in ocular micrometer scale units.

m = mean

σm = standard deviation of the mean

d = difference between means of adjacent intervals

σd = standard deviation of the difference

^a Measurements in this experiment were taken with a different set of lenses than those used in the subsequent experiments. Absolute values are therefore not comparable to those of other series.

an interference of the nuclear swelling by phosphate possibly by dissolution of some nuclear constituents and inhibition of the phosphate effect by citrate. Whether or not the effect of the citrate is the result of possible competition with phosphate for nuclear constituents cannot be determined from these data although it is suggestive that the nuclear diameter is reduced by a factor of 2 when the ratio of phosphate to citrate is increased by a factor of 2.

As shown in Table IV, there is in the phosphate buffered solutions, at pH 7.4, a range of phosphate concentrations (6.5×10^{-5} - 6.5×10^{-4}) in which the effect of phosphate is minimal, i.e., the swelling in this range was greater than at lower and

TABLE II. *Mean nuclear diameters in 0.065 M MacIlvaine buffer in 4.8 M glycerol.*

Molarity citrate	0.011	0.009	0.006	
Molarity phosphate	0.107	0.113	0.118	control
pH	6.98	7.20	7.40	
Mean diameter	20.4	13.0	10.1	9.6
Fraction $\leq$ control + 1	1/20	4/18	15/20	

TABLE III. *Mean nuclear diameters in 0.065 M phosphate buffer in 4.8 M glycerol.*

pH	6.90	7.10	7.19	7.30	7.39	control
Mean diameter	13.2	9.6	9.5	10.6	9.0	9.6
Fraction $\leq$ control + 1	7/20	18/20	6/8	6/10	16/20	

TABLE IV. *Mean nuclear diameters in relation to phosphate buffer concentration in 4.8 M glycerol.*

Phosphate concentration $\times 6.5$	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Mean nuclear diameter	12.4	14.9	13.8	12.3
Standard deviation of mean	0.75	0.82	0.90	0.73
$\sigma d/d$		2.25	0.90	2.24

higher concentrations. From these observations we conclude that phosphate plays a significant role in determining the capacity of the nucleus to swell and the extent of the swelling which may occur in hypertonic glycerol solutions. Whether, as the data of Table IV suggests, this may be through phosphate involvement with metabolic activity of nuclear constituents as, for example, by its relation to ATP or other organic phosphate and pyrophosphate compounds remains to be determined. Although this suggests possible similarity to mitochondrial swelling, comparison of the action of Ca^{++} and Mg^{++} on the swelling of these nuclei in glycerol have shown no difference in the effects of these ions.

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THE FINE STRUCTURE OF THE SEX CHROMATIN BODY¹

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SINCE the discovery of the sex chromatin by Barr and Bertram in 1949 [1] a host of literature, which I have reviewed recently [11], has appeared on this subject. It was proposed by Barr and his co-workers [2, 10, 14] that the sex chromatin of the female nucleus is the result of the fusion of the heterochromatic portions of the two X-chromosomes. This is analogous to findings in insects [6]. I could show, in fact, that at times the sex chromatin was composed of two portions [11]. Other workers have mentioned that the sex chromatin shows certain shapes and divisions [3, 4, 5, 10, 18]. Crouch and Barr [5] suggested the term "diplococcus" for the split sex chromatin.² The bipartite form of the sex chromatin should not be confused with the "double sex chromatin", which I have described previously [11] and which represents two complete and widely separated sex chromatin bodies within one nucleus. These double, occasionally multiple bodies are found in nuclei which may be in a polyploid state or in the process of endomitosis. This latter phenomenon will soon be reported on in greater detail. Barr and Moore [3] interpret the double forms which they found in tumors, as the expression of abnormal mitoses with a doubling of the chromosomal complement.

With the aid of an improved staining procedure [12] I decided to determine whether the bipartites are only chance occurrences or whether the sex chromatin really consists of two parts.

Materials and Methods.—The sex chromatin was studied systematically in the following human tissues: whole mounts of amnion and chorion laeve from embryos of various developmental ages, as well as from hydatidiform moles; smears from oral, vaginal and urethral mucosa; sections from intestinal and nasal epithelium, decidua, ovaries, skin and nervous tissue. The tissues were fixed within 5 to 50 min. after removal or death in modified Davidson's fluid and 95 per cent alcohol. Embryonic membranes and mucosal smears were prepared as described in a previous publication [12]. The paraffin embedded tissues were cut 5 and 10 μ thick and all preparations were stained according to the Feulgen and thionin methods described in the above cited paper. Hydrolysis times of 20, 40, 50 and 60 min. in 5 N HCl at 20°C were used. Some tissues were also cut 2 and 5 μ thick and mounted unstained for phase contrast microscopy.

¹ This paper was presented in part at the "Symposium on Nuclear Sex" at the Kings College Hospital Medical School, London, September 6-7, 1957.

The author wishes to thank Prof. G. Wolf-Heidegger, Director of the Department, for the help which made this work possible.

² In order to avoid the use of a generic name for an intranuclear structure, the diplococcus of Crouch and Barr will be called the "bipartite" form of the sex chromatin.

On each of the tissue types the frequency of the bipartite sex chromatin was determined by counting the number of these forms which could be found in 500 nuclei containing sex chromatin. In addition 100 bipartite sex chromatin bodies in the nuclei of the connective tissue and epithelium of amnion and chorion, as well as in decidua, were measured for total size, component size and for the distance between the two components with an R. Winkler ocular micrometer at a total magnification of $1200\times$.

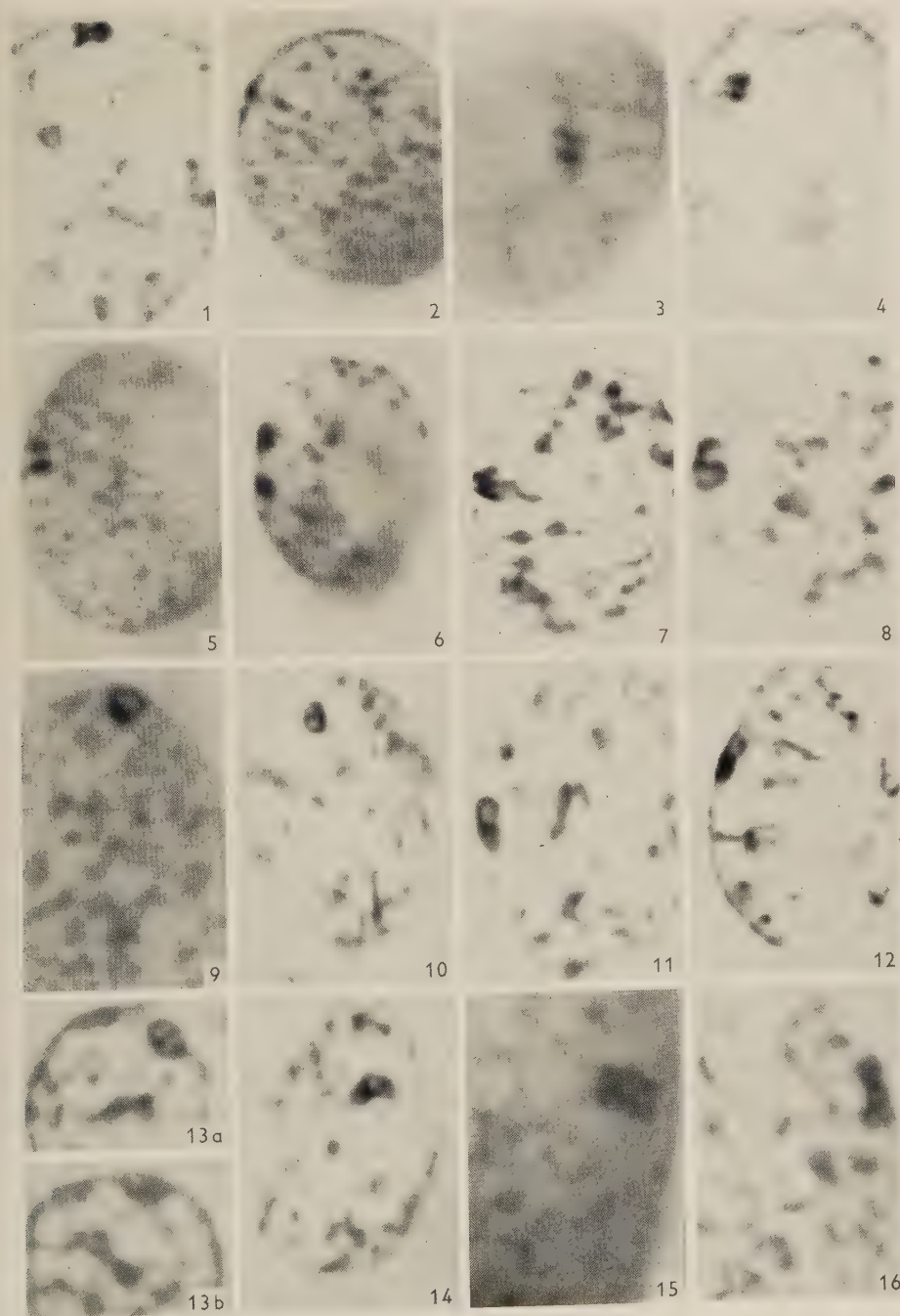
Observations.—Aside from the various shapes of the sex chromatin, which have already been reported by various workers, I was able to recognise details of internal structure. The most common form of the sex chromatin is the bipartite in which two small masses, ranging 0.3 to 0.7μ (average 0.5μ) in their largest dimension, lie close to each other separated by a distance which, when measurable, ranges between 0.2 and 0.5μ (average 0.3μ). Such forms are seen in Figs. 1, 2, 3, 4 and 5. The total size of such a bipartite ranges between 0.8 and 1.4μ , averaging 1.1μ . This is the order of size which has been generally reported for the non divided sex chromatin [10, 13–17]. The individual chromatin bodies of a double sex chromatin as shown in Fig. 6, are each as large as a non divided sex chromatin. In addition the distance between the components of a double form may be as large as the nucleus is long. Of all the sex chromatin bodies counted in embryonic membranes 13.5 per cent were of the bipartite form. The two masses may be homogenous or one or both parts may be vacuolated as shown in Figs. 10, 11, 12, 13*a* and *b*. The latter two illustrations were photographed in phase contrast. The appearance here is not essentially different from the photographs taken from material which was hydrolyzed and stained according to the thionin or Feulgen methods. Although the bipartite could be found in all female tissues studied, the frequency with which it appears varies greatly according to tissue type. It is most commonly found in the embryonic membranes examined. The fact that these tissues can be examined without the need of embedding and sectioning procedure may, in part, explain this phenomenon. Oral, vaginal and urethral mucosa smears, however, are also prepared in this way and yet the bipartite form is rare in these nuclei. This may be due to the fact that in the latter nuclei the sex chromatin appears as if it were tightly pressed against the nuclear membrane so that any division is obliterated (Fig. 2).

Aside from the bipartite form further details of fine structure can be recognized in many chromatin bodies, particularly in the nuclei of the embryonic membranes which had been hydrolyzed for a long time. Often the sex chromatin appears as a small $\bigcirc$ as shown in Fig. 9. Sometimes multiple vacuoles are present which range in number from two to seven (Figs. 10 through 16). At times the sex chromatin may show a spiral like form which, when projected onto a single plane, is suggestive of the letter "W" or "S" (Figs. 7 and 8). Chromatin strands sometimes emerge from

Except figs. 2, 13*a* and *b*, all the nuclei shown are from whole mounts of amnion or chorion. Fig. 2 is from a smear of the oral mucosa. Figs. 13*a* and *b* are from 5μ sections of the nasal mucosa photographed in phase contrast.

Figs. 5 and 11 are from preparations stained according to the Feulgen method. All the others are from thionin preparations. Figs. 8, 9, 15 and 16 are reproduced at a magnification of $5300\times$, all others at $4000\times$.

For further explanations of the illustrations see the text.



such structures (Fig. 7). In preparations stained according to the Feulgen method, with light green as a counterstain, a small green nucleolus-like body was often found adjacent to the red sex chromatin.

Discussion.—The observed finer structure of the sex chromatin strongly suggests that this body is composed of two portions. It must be remembered that the separate components of the bipartite are separated by a distance not much greater than that which can be resolved with the light microscope. The orientation of the sex chromatin in relation to the optical axis is an important factor in the consideration of frequency with which the bipartite can be resolved. If 13.5 per cent of the sex chromatin bodies in the embryonic membranes show this division, then it is very possible that all sex chromatin, at least in these tissues, are of the bipartite form. The other details of fine structure are reminiscent of the structure described for the large chromosomes of plants and animals. The sex chromatin not only resembles the chromosomes morphologically but histochemically as well [11]. All this evidence plus the observations [7, 8, 11] that the sex chromatin is independent of environmental factors, strongly supports the view of Barr and his co-workers that the sex chromatin represents the heterochromatin of the X-chromosomes. In the intermitotic nucleus the two heterochromatic rests from the XX complement of the female nucleus fuse to form an apparently single body, which as I have shown, can again be resolved into two portions. One would expect, therefore to be able to find a single half sized structure in the male nucleus. Such a body can in fact often be seen in male nuclei [2, 5, 11, 13], but normally its extremely small size make its recognition and differentiation from the rest of the particulate chromatin difficult.

At times the vacuoles that are seen in association with a sex chromatin stain green with the light green of the Feulgen reaction. (See color plate III fig. 1 c in [11].) This observation suggests that at times a small nucleolus may be associated with the sex chromatin. Should this be true, then it would in no way contradict the proposed theory as to the origin of the sex chromatin, since it is well known that the chromosomes and the nucleoli are intimately connected during the process of cell division. It would therefore not be surprising to find that a heterochromatic chromosome should maintain its nucleolus or at least nucleolar portion. In this respect the original theory of Barr and Bertram [1] that, "the nucleolar satellite (sex chromatin) may be derived from the portion of the nucleolar chromosome known as the nucleolar organizer" may still hold true, even though these authors have since refuted this theory for the one discussed above. It is not impossible that both theories are in part appropriate. Further studies are needed before these views can be validated.

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INHIBITION OF HATCHING IN THE SEA URCHIN, *LYTECHINUS* *VARIEGATUS*, BY TRYPTOPHANE¹

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UPON fertilization, a membrane elevates from the surface of the sea urchin egg. Subsequently, this fertilization membrane dissolves to liberate the free swimming blastula larva. This membrane dissolution apparently results from the action of an agent, the "hatching enzyme". Ishida [2] obtained solutions of the hatching enzyme by centrifuging down hatching blastulae (*Strongylocentrotus* sp.) and decanting the supernatant. He found that eggs fertilized in such a solution failed to develop fertilization membranes and that fully formed membranes dissolved in the solutions. Kopac [3] obtained similar results using *Arbacia* eggs. The following observations concerning the inhibiting action of tryptophane on the dissolution of the fertilization membrane are submitted as a contribution to the study of the hatching process.

Eggs of the sea urchins *Lytechinus variegatus* and *Arbacia punctulata* were used. Solutions of DL-tryptophane ranging from $10^{-1}M$ to $10^{-5}M$ in ten-fold dilutions were prepared in sea water and adjusted to pH 8. Freshly fertilized eggs were placed in

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the solutions and permitted to develop. $10^{-1}M$ tryptophane proved to be toxic to the eggs, but at lower concentrations (optimum $10^{-2}M$ in the summer and $10^{-4}M$ in the winter) development proceeded normally to the hatching stage. However, at the blastula stage dissolution of the fertilization membrane failed to occur in the majority of cases. In those cases in which hatching did occur, the membranes remained easily visible on the bottom of the dish even in concentrated groups of blastulae, whereas the membranes of controls were completely dissolved in a short period of time. The blastulae which failed to hatch continued to develop within their membranes. Pigment formed in the larvae, but within the confines of the membrane arms failed to develop normally. The skeleton was very abnormal and development ceased with the death of the larvae about 15 hours after the normal hatching time.

The inhibition of dissolution of the fertilization membrane could result from one of several effects of tryptophane. Tryptophane could act by rendering the membrane substrate inaccessible to the hatching enzyme; it could act on the enzyme itself by blocking its action or preventing its formation or it could remove some essential ion from the sea water by virtue of its metal binding action. The last of these possibilities appears to be unlikely since metal binding agents do not produce this effect on sea urchin development [6] and since other amino acids (glycine, alanine, phenylalanine and tyrosine) did not prevent hatching of *Lytechinus* eggs. Furthermore, the action of tryptophane appears to be rather specific since other related substances (indole, indole-3-acetic acid, indolpropionic acid) also failed to inhibit membrane dissolution. Attempts to distinguish between the first two possibilities have so far failed, due to the fact that all attempts to prepare active solutions of *Lytechinus* hatching enzyme have been unsuccessful. Accordingly, repetition of the experiments using *Arbacia* material was begun. However, tryptophane failed to inhibit membrane dissolution in this form. This result at least suggests that the action of tryptophane is rather specific for *Lytechinus*. Finally, active solutions of *Arbacia* hatching enzyme failed to affect the fertilization membranes of *Lytechinus* eggs.

Aside from the interesting and apparently rather specific action of tryptophane on membrane dissolution, these observations confirm the results of others [1, 4, 5] which show that the composition of sea urchin fertilization membranes vary considerably from species to species.

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STUDIES ON PRODUCTION OF HYALURONIC ACID IN TISSUE CULTURE

THE PRESENCE OF HYALURONIDASE IN EMBRYO EXTRACT¹

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On addition of a few drops of 1 N acetic acid to 1 ml sample containing highly polymerized hyaluronic acid and proteins, a tight mucin clot in a clear solution is formed. The mucin clot formation can be prevented by hyaluronidase (Fig. 1). The test is specific for hyaluronic acid present in native fluids, or produced in tissue culture [3-6]. It is negative for chondroitin sulfates and for hyaluronic acid obtained by isolation. Chick embryo extract prepared without saline gives a tight mucin clot and contains 210 to 240 mcg hyaluronic acid per ml. The regular medium used in present experiments containing 20 per cent of half diluted embryo extract, contained about 20 mcg hyaluronic acid per ml, which is below the minimal concentration for mucin clot formation. Concentrated embryo extract (70 to 100 per cent EF₅₀) gives a tight mucin clot.

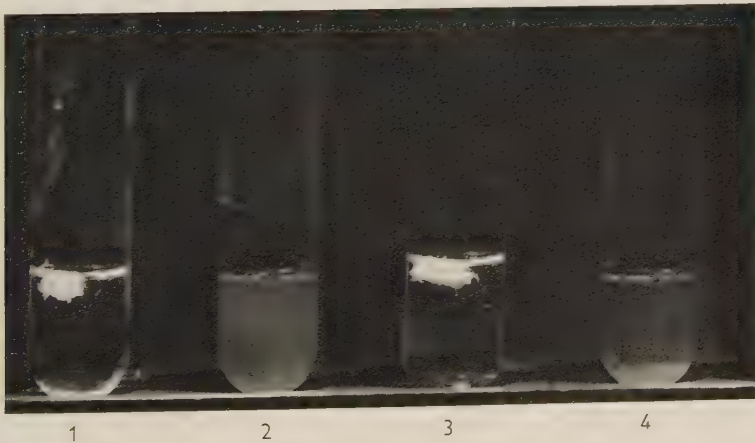


Fig. 1.—1 and 3 positive mucin clot test; 2 negative mucin clot test; 4 prevention of mucin clot formation by hyaluronidase.

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The starting point of the present investigation was the observation, that if concentrated chick embryo extract was used as culture medium, the supernatant fluid of living cultures of mesenchymal tissues gave a tight mucin clot, while the supernatant fluid of dead cultures (as evidenced by absence of vital staining) gave no mucin clot, although the medium before incubation gave a positive mucin clot test. Evidently the hyaluronic acid originally present in the embryo extract was depolymerized in the time intervals between feedings. This conclusion was confirmed when concentrated embryo extract failed to give a mucin clot after incubation at 37°C for a few days without cultures. The failure to produce a mucin clot was not due to the presence of proteolytic enzymes, because addition of serum did not restore the quality of embryo extract to form a mucin clot after incubation. It has been thought that chick embryo extract may contain small amounts of enzyme liberated by autolysis sufficient to hydrolyze in a few days hyaluronic acid originally present in concentrated embryo extract. The low concentration of enzyme and the fact that it is used up first by hydrolyzing hyaluronic acid contained in embryo extract, was thought to be the reason why it does not also depolymerize the hyaluronic acid subsequently produced in tissue culture.

Methods.—Chick embryo bone and heart, rat subcutaneous tissue, and rat tail tendon were used. The medium consisted of 20 per cent embryo extract, 30 per cent horse serum and 50 per cent amniotic fluid [2]. Change of medium was carried out every 5 to 6 days. The presence of hyaluronidase was tested by the mucin clot prevention method after incubation at 37°C for various periods of time. In order to determine, whether low molecular weight substances such as ascorbic acid, or cysteine were responsible for the depolymerization, samples of embryo extract were dialyzed against 0.15*N* NaCl at 4°C for various periods of time, after the method of Balazs and von Euler [1]. No difference was observed in depolymerizing activity of dialyzed and non-dialyzed samples.

Results and conclusions.—In Table I results of 3 experiments are summarized. The positive mucin clot test in 70 and 100 per cent embryo extract became negative after 5 days of incubation with dead cells (in absence of vital staining), while such media as well as regular media incubated with living and hyaluronic acid producing cells yielded a mucin clot after 5 days of incubation. Thus, regardless of the concentration of chick embryo extract a positive mucin clot test indicates production of highly polymerized hyaluronic acid by living cells *in vitro*. Table II demonstrates the depolymerization of hyaluronic acid in undiluted embryo extract after incubation at 37°C without tissue. In Table III the supernatant fluids containing hyaluronic acid produced in tissue culture and subsequently incubated at 37°C for periods of time of 4 to 55 days still gave a positive mucin clot test in 93 per cent of tubes. The loss of the quality of mucin clot formation was the same when extract was prepared from embryos deprived of liver, spleen, or eyes. The method can also be applied to single organs of the embryo.

Hyaluronic acid produced in tissue culture evidently is not hydrolyzed by the enzyme contained in concentrated embryo extract. The assumption, that hyaluronic acid newly produced in tissue culture is in some way protected against low concentrations of enzyme present in embryo extract is not substantiated. The most probable reason for the absence of depolymerization of hyaluronic acid produced in tissue culture appears to be the fact that the enzyme had been used up in the hydrolysis of hyal-

TABLE I. *Production of hyaluronic acid in tissue culture and hyaluronidase activity of chick embryo extract.*

# of tubes	State of cultures before experiment	Medium	Mucin clot formation before incubation	Mucin clot formation in culture fluid after 5 days incubation	State of culture
12	Very good growth	regular ¹	No mucin clot	Mucin clot +	Very good growth
7	Fair to good	70 % EE ²	Mucin clot +	Mucin clot +	Good growth
12	Dead	70 % EE ²	Mucin clot +	No mucin clot	Dead
4	Dead	regular ¹	No mucin clot	No mucin clot	Dead
2	Dead	100 % EE	Mucin clot +	No mucin clot	Dead

¹ 20% EE, 30% horse serum, 50% amniotic fluid.² 70% EE, 15% horse serum, 15% amniotic fluid.TABLE II. *Incubation of concentrated chick embryo extract at 37° C.*

# of samples	Before incubation	Days of incubation	No mucin clot
12	Mucin clot +	1	9
12	Mucin clot +	2	10
10	Mucin clot +	3	9
7	Mucin clot +	4	7

TABLE III. *Incubation of supernates from tissue cultures.*

Tissue	Medium tested before feeding	After 5 days of growth	Days of incubation of supernates alone at 37° C	# of samples	Mucin clot	No mycin clot
Rat sub cutan.	No mucin clot	Mucin clot +	4 to 18	9	9	—
Chick embryo bone	No mucin clot	Mucin clot +	8 to 33	41	38	3
Rat tail tendon	No mucin clot	Mucin clot +	4 to 55	7	6	1

uronic acid of the embryo extract. Attempts to depolymerize hyaluronic acid in synovial fluid and in supernates from chick bone tissue cultures by incubation with embryo extract gave negative results, evidently because the low enzyme concentration in embryo extract was used up first by digestion of hyaluronic acid present in embryo extract.

It is inferred that in embryo extract hyaluronidase is gradually liberated by autolysis and depolymerizes hyaluronic acid present in embryo extract. The hyaluronidase concentration is probably below 0.01 TRU per ml, as estimated by incubation for

various periods of time of synovial fluid of known hyaluronic acid concentration with hyaluronidase (Wyeth) after testing its mucin clot prevention activity. Embryo extract represents thus a system containing both substrate and enzyme set free by autolysis, which act on each other. The concentration of the enzyme can be estimated by determining the time required for depolymerization of a known hyaluronic acid concentration in embryo extract. Hearts from 13 to 15 days old chick embryos were also used for preparation of extract. 80 to 90 hearts, including large vessels, were used in each of 3 preliminary experiments. The extracts have been shown to contain hyaluronic acid in concentrations of about 45 to 75 mcg/ml, and also hyaluronidase in low concentration.

Summary.—1. The formation of a tight mucin clot with acetic acid that can be prevented by hyaluronidase, indicates the presence of highly polymerized hyaluronic acid. Cultured cells of various mesenchymal tissues produce *in vitro* highly polymerized hyaluronic acid. 2. Freshly prepared, concentrated embryo extract contains hyaluronic acid, the concentration of which was estimated to be about 210 to 240 mcg/ml. 3. Hyaluronic acid present in concentrated chick embryo extract was depolymerized when incubated at 37°C for 1 to 4 days. Chick embryo extract may contain small quantities of free hyaluronidase, which slowly depolymerizes hyaluronic acid at 37, and more slowly at 4°C. 4. In tissue culture with several days' intervals between changes of medium, hyaluronic acid contained in embryo extract was depolymerized. 5. Culture fluid containing hyaluronic acid produced in tissue culture subsequently incubated for up to 55 days, did not lose the quality of mucin clot formation.

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ACTION OF RIBONUCLEASE ON YEAST PROTOPLASTS

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THE degree of penetration of ribonuclease into living cells varies widely depending on the type of cell being studied. Brachet [2, 3] and Kaufmann and Das [8] have shown that *Amoeba* and onion root tips are sensitive to ribonuclease action. Furthermore, permeability to ribonuclease *in vivo* has been demonstrated by Chèvremont and Chèvremont-Comhaire [7] in fibroblasts, by Ledoux, Le Clerc and Vanderhaeghe [9] in amphibian eggs, and by O'Hern and Henry [6] in cells of *Coccidioides immitis*. On

the other hand, Beljanski [1] and Brenner [4] have found that the surface of living microbial cells is impermeable to ribonuclease. The results of Lester [10], Bridoux and Hanotier [5] are also important; they have shown that intact cells of *M. lysodeikticus* are impermeable to ribonuclease, whereas naked protoplasts obtained by the action of lysozyme are permeable. Furthermore, conflicting results concerning the action of ribonuclease on metachromatic (volutin) granula in bacterial cells have been obtained. According to Minck and Minck [12], metachromatic granula disappear in fixed *Cl. diphtheriae* cells under the influence of ribonuclease. Wachstein and Pisano [17] were unable to verify this result.

In our experiments we have compared the action of ribonuclease on intact living yeast cells with the action of ribonuclease on the development of naked yeast protoplasts. Furthermore, we have followed the influence of ribonuclease on volutin granula in fixed normal cells and in protoplasts during various stages of their development.

The strain of baker's yeast used in these experiments was obtained by isolation of one cell. Naked protoplasts were prepared by the author's method [13, 14] which involves the enzymatic lysis of the cell wall. The development of yeast cells and protoplasts was followed in moist microscopic chambers on agar films with synthetic medium. Solutions of crystalline ribonuclease (kindly supplied by Dr V. Liebl) were applied in a concentration of 1 mg/ml at pH 5.4–6. An alcoholic solution of methylene blue was used to stain the volutin after the cells had been fixed in 96 per cent alcohol.

Yeast reproduction is not influenced by ribonuclease even after 48 hours' action. Cytologically the treated cells are not different from the control samples. After fixation and staining (following inactivation of the ribonuclease in the medium) the treated cells retain their volutin granula in the cytoplasm. However, if the cells are first fixed and then treated with ribonuclease, the effect of the treatment is pronounced. After 4 minutes' treatment (culture 24–48 hours old) all volutin granula disappear. This can be followed directly by the microscope (Fig. 1*a, b*).

During the first 12 hours naked yeast protoplasts inoculated on a culture medium with ribonuclease develop in quite the same way as the control samples and large vacuolised formations occur [15]. Later on further structural changes take place and plasmatic formations, able to regenerate as cells, arise (Fig. 2*a*). In cultures with ribonuclease, however, all the formations do not develop in this manner. Usually their development stops after 12 hours in the stage of vacuolisation. A smaller part of the plasmatic formations completes the normal development. During the first 12 hours the developing protoplasts contain a large amount of volutin, mostly in vacuoles, both in the medium containing ribonuclease and in the control samples (Fig. 2*a*). After fixation followed by 10 min. action of ribonuclease, the metachromatic granula (Fig. 2*b*), as well as the vacuolar metachromatin, disappear completely.

It may be assumed, according to these results, that ribonuclease does not penetrate into intact living cells. Ribonuclease action on volutin is visible after only 10 minutes' treatment after fixation. On the other hand naked protoplasts (depending possibly on their stage of development) are partly permeable to ribonuclease. But ribonuclease penetration is very slow, since its effect on protoplast development does not appear until approximately 12 hours after the beginning of the treatment. There is great individual variability in the protoplast permeability for ribonuclease. Some develop quite normally, which indicates that the ribonuclease has not penetrated.

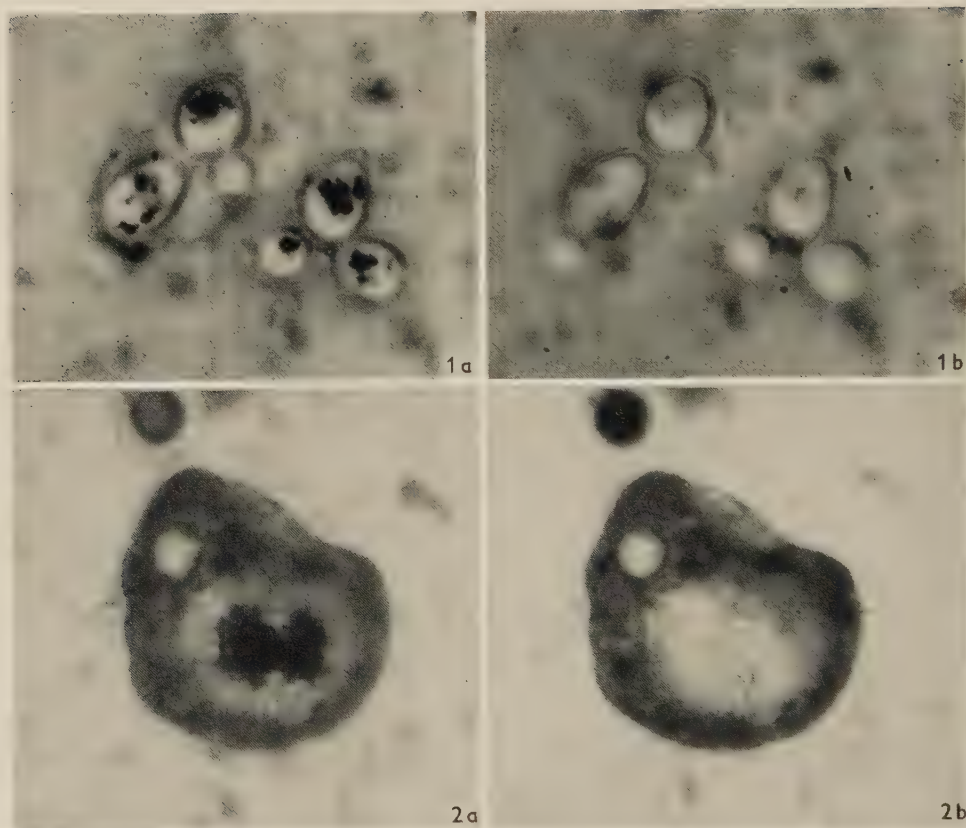


Fig. 1.—(a) Yeast cells with volutin granula (stained by toluidine blue after fixation with alcohol); (b) the same cells after 5 min. ribonuclease treatment ($\times 200$).

Fig. 2.—(a) Plasmatic formation with volutin precipitated in the vacuole (toluidine blue after fixation with alcohol); (b) the same object after 5 min. ribonuclease action ($\times 1500$).

Our experiments have proved that plasmatic volutin is made up of ribonucleic acid [6]. Moreover, ribonuclease action causes the volutin contained in the vacuoles to disappear. Up to now volutin has been assumed to be formed from storage metaphosphate only [18, 11]. We find it necessary to assume that nucleic acid is also present in the structures of vacuolar volutin.

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A NEW TISSUE CULTURE FLASK WITH DEMOUNTABLE BOTTOM¹

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ONE of the objections to standard tissue culture flasks of the long-necked type is the inaccessibility of the tissues for microscopic examination. Gross examination under low or medium magnification is possible through the bottom of such flasks, but close study under oil immersion lenses is not practical, especially with phase contrast equipment. Use of inserted coverslips limits the examinable substrate to the area of a coverslip small enough to pass through the neck, i.e. about 11×22 mm or less. The occurrence of growth on both faces of inserted coverslips also presents a troublesome problem in wet as well as with permanent mounts.

Jacoby [1] has partly surmounted these limitations by using a large type Carrel flask of 5 cm diameter, with a wide (30 mm diameter) wax-sealed window in the top, through which comparatively large coverslips could be removed for examination. Zuckerman [2] solved the difficulty in a slightly different manner by cementing large (40 cm diameter) round coverslips onto the open face of Carrel flasks from which the bottom had been removed. Following fixation of contained tissue cultures, the bottom was soaked off in alcohol.

Our problem, involving the *in vivo* study of the exoerythrocytic phase of avian malarial parasites in tissue culture, placed further demands on type of substrate and its handling. *In vivo* study dictated the use of phase contrast microscopy and wet mounts; however, the small removable coverslips customarily used with Porter

¹ The opinions and statements contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

flasks present a definitely limited surface for observation. Then, too, long term observations of *in vivo* material demands more or less continuous perfusion with nutrient media if growth of the host cell and contained parasite is required.

The fragile walls of standard Carrel or Porter flasks were found unsuitable for use with a pressure-sealed gasket for the removable bottom. Instead, it was found necessary to devise a special type of culture flask which would meet all requirements. This flask was made from standard Pyrex tubing (outside diameter 35 mm, wall thick-

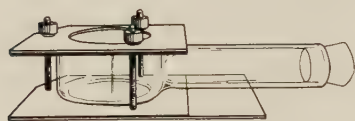


Fig. 1 (left).—The assembled flask.

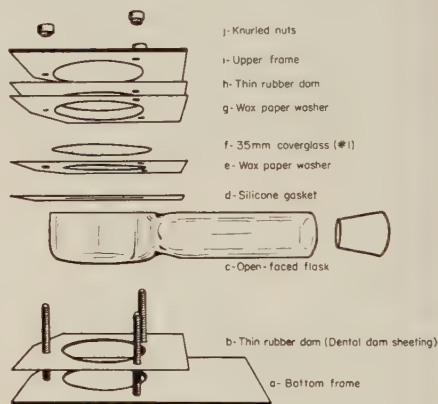


Fig. 2 (right).—Exploded view of flask assembly.

ness about 1.5 mm). One end of a length of such tubing was sealed with oxygen flame and flattened. The other end was drawn out to a point, leaving a straight wall of 5 to 6 cm above the bottom. A hole of about 12 mm diameter was then blown in the side wall near the flat bottom, and a 5 cm piece of Pyrex tubing (outside diameter 16 mm) attached at this point and at right angles to the wall. Annealed at 570°C, the excess of 35 mm tubing was later cut off on a glass-cutting wheel or diamond saw, so as to give a wall depth of 17 mm from flat bottom to the cut edge. The edge was then ground perfectly flat on the face of the glass-cutting saw so as to provide a true seating for the gasket seal. Properly executed, this flask gives a vessel of about the same volume as a Porter flask, but one which is more durable and much more easily cleaned, and for special purposes, more versatile.

In use, the flask is fitted with a #1, 35 mm round coverglass as a demountable bottom, which is held in place in a stainless steel frame. (See Fig. 1, the assembled flask.) The cultures are incubated with the flask in the inverted position, that is, with the coverglass side down, so that the growing cells spread out over the coverglass bottom. Prevention of leakage is realized by use of silicone rubber gaskets (West Rubber Co., Phoenixville, Pa.) about 1 to 2 mm thick, which proved for all practical purposes, nontoxic for our cultures. Assembly of the flask is seen in Fig. 2, an exploded view showing the different components in superimposed position. To lessen a tendency of the rubber gaskets to stick, wax paper washers are placed on either side of the coverglass. Thin rubber dams separate the bottom and top of the flask assembly from the metal frames, preventing slippage and cushioning the edge of the coverglass, lessening breakage when the frame is tightened.

The use of this type of flask offers the following advantage for tissue culture work: (1) The 35 mm coverglass bottom presents an available area which is much greater than that afforded by the small coverslips commonly employed with Porter flasks. (2) The demountable bottom may be viewed either in temporary mounts after removal from the flasks, or may be fixed and stained and used as permanent mounts. For the latter purpose, large 2" × 3" pathology slides, circled with warmed vaseline on a slide ringing stage, serve as a convenient base. (3) Growth occurs only on one face of the coverglass, obviating the need to clean the other face if it is to be used as a wet mount. (4) Such a mount presents a single coverglass between microscope objective and the tissue, whereas double coverslip preparations interpose two. This reduction in the number of surfaces has been found to be an appreciable advantage with phase contrast microscopy. (5) The comparatively large area of the preparation, besides giving additional choice of fields, also encloses a greater quantity of culture media, which provides a temporary mount of longer lasting qualities before evidence of degeneration becomes apparent. (6) This flask makes possible the use of gassing with carbon dioxide, a procedure not possible where cultures are grown in Rose chambers from the start. (7) Finally, this coverglass may be mounted directly in a Rose chamber after the tissue is well grown out, and used as a perfusion preparation

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POLARITÉ DE LA CENTROSPHÈRE ET DES CORPS DE GOLGI DANS LES LEUCOCYTES DES MAMMIFÈRES

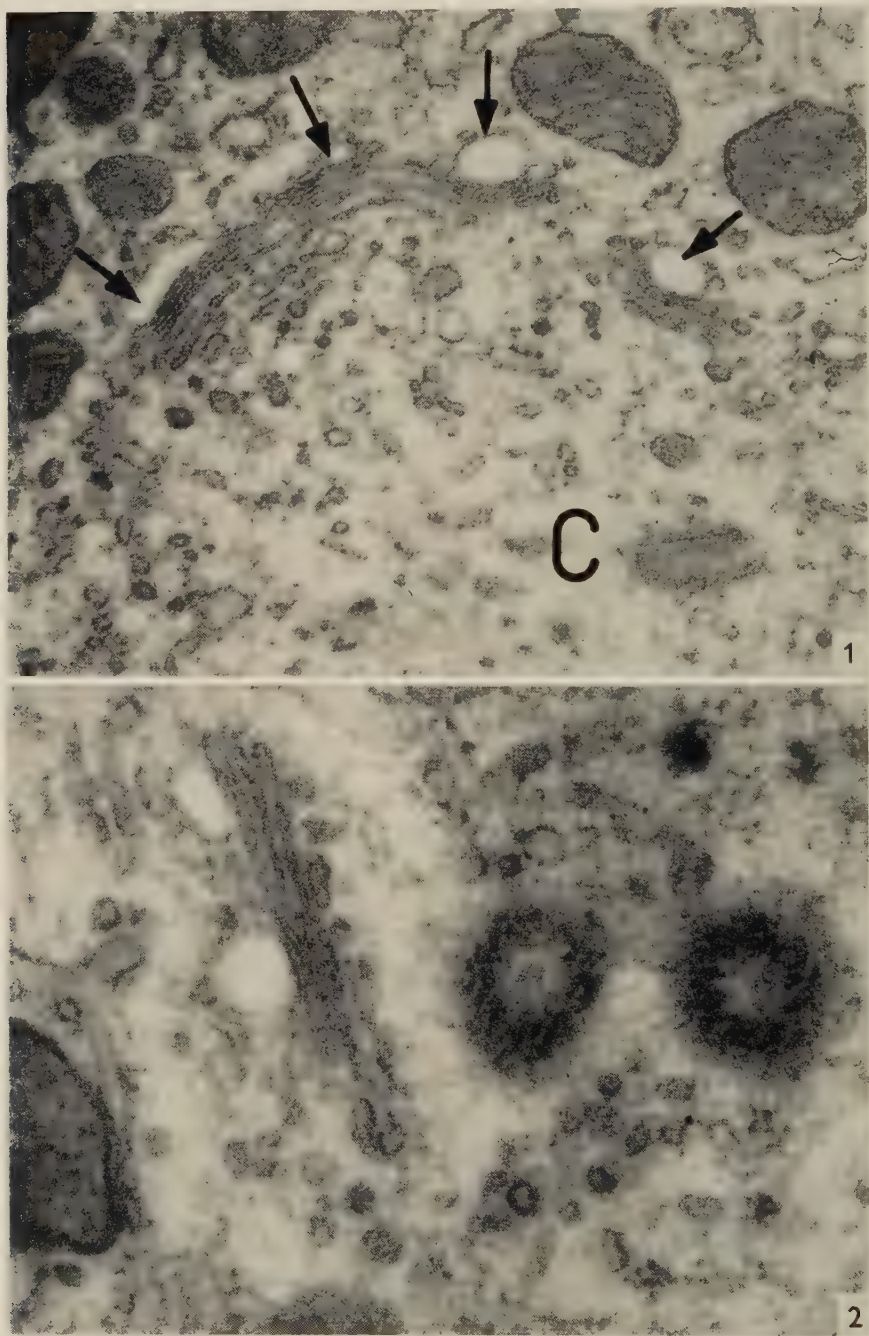
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DANS des recherches récentes, P. P. Grassé a montré, dans les spermatides d'escargot et chez certains infusoires, le fait intéressant d'une polarité très nette des corps de Golgi par rapport aux dérivés filamenteux du centrosome [2]. Chaque dictyosome est une pile de saccules clos et aplatis constituant la substance chromophile des auteurs classiques. La substance chromophobe est formée de vésicules de grande taille qui se disposent toujours contre la face distale (par rapport au filament parabasal, émanation du centrosome).

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Dans les leucocytes de mammifères (granulocytes, plasmocytes, monocytes) on peut voir au microscope électronique une polarité analogue mais par rapport au centriole lui-même.

Dans ces cellules, la centrosphère possède une disposition très particulière; elle est constituée par une masse sphérique de cytoplasme, sans mitochondries, sacs ergastoplasmiques ou granules. Elle est relativement pauvre en eau et riche en matières protéiques, si bien qu'après dessiccation et ombrage elle apparaît, sur des leucocytes étalés, comme une sorte de colline en saillie sur le reste du cytoplasme. D'autre part, sur les leucocytes vivants examinés par micro-cinématographie en contraste de phase, la sphère montre des oscillations rythmiques très caractéristiques [4].

Au centre de la sphère se trouvent deux centrioles [1].

Les corps de Golgi se répartissent à la périphérie de la centrosphère [5]. Ils sont disposés généralement d'une manière régulière. Toutefois, ils peuvent former des plissements. Dans ce cas, l'interprétation des images données par les coupes présente quelques difficultés. Chacun de ces corps de Golgi comporte essentiellement un paquet de six à huit sacs aplatis (dictyosomes de Perroncito). Sur leurs pourtours ces sacs paraissent donner naissance par bourgeonnement à de fines vésicules, décrites par Grassé [3]. Sur la face distale du dictyosome, c'est-à-dire *sur la face opposée à l'ensemble centrosphère-centriole*, s'observent de grosses vacuoles à contenu clair, semblant correspondre à la « substance chromophobe » de la cytologie classique. Ces vacuoles paraissent résulter de la dilatation et du gonflement des sacs les plus distaux du dictyosome. Sur la face proximale, c'est-à-dire du côté de la centrosphère, une telle vacuolisation ne s'observe pas. Dans les leucocytes de mammifères, il semble donc raisonnable de parler d'une polarité de l'appareil de Golgi par rapport aux centrioles.

Les figures 1 et 2 donnent des exemples de ces dispositions.

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Fig. 1.—Une partie de la centrosphère d'un plasmoblaste humain. On voit les dictyosomes répartis à la périphérie de la centrosphère. Les flèches montrent les vésicules distales (chromophobes). Le centriole (C) est coupé longitudinalement.

Fig. 2.—Deux centrioles coupés transversalement et un corps de Golgi avec petites vésicules proximales et grosses vésicules chromophobes distales.

PRESENCE OF DNA SYNTHESIS AND MITOSIS IN THE BRAIN OF YOUNG ADULT MICE¹

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CELL division is generally considered to be rare or absent in the nervous system of adult animals, except under pathological conditions [4, 6]. However, Allen in 1912 had described the presence of cell divisions in the central nervous system of the adult rat [1]. Radioautographic observations in mice after injection of C¹⁴-adenine—a possible precursor of DNA—had indicated that some cells of the nervous system may synthesize DNA and divide [7]. It was therefore decided to reexamine the problem using thymidine, a specific precursor of DNA [2, 5]. The thymidine used was labelled with tritium because of the high resolution obtainable in tritium radioautographs.

One hundred μ c of tritium labelled thymidine (118 mc/millimole) dissolved in 0.45 ml saline were injected into each of three young adult CFW mice weighing 13–14 g, which were sacrificed 8, 24 and 72 hours later respectively. Various parts of the nervous system and other tissues were fixed in Bouin, embedded in paraffin, sectioned at 6 μ and radioautographed by the coating technique [3].

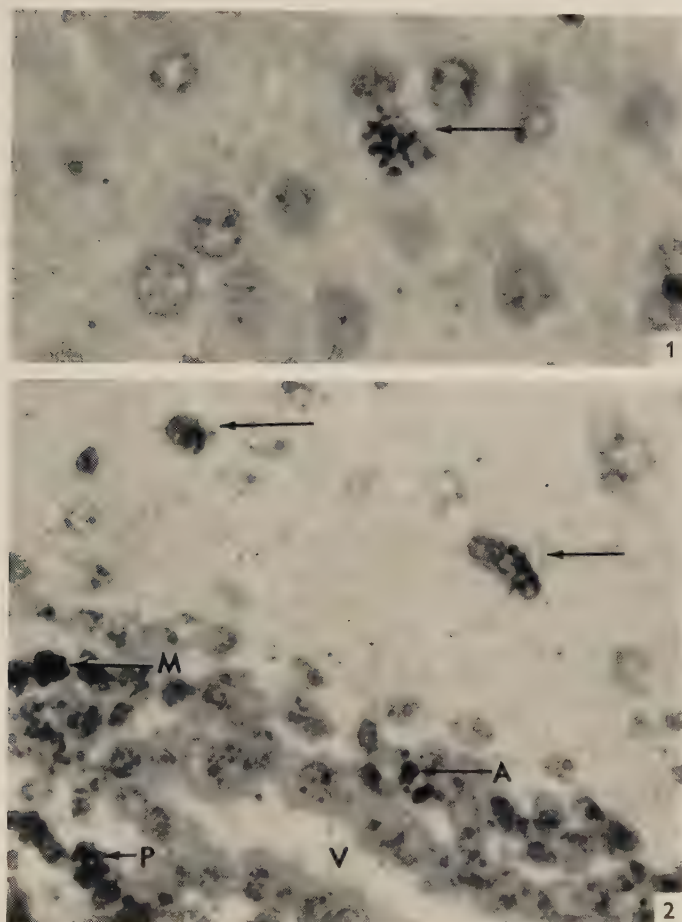
Radioautographic reactions were observed at the three time intervals. In all tissues examined the reactions overlay nuclei. In the cerebrum, reactions were most frequent in clusters of cells underlying the ependyma of the lateral ventricle (about 10 per cent of the nuclei being reactive at the 8-hour interval). These cells correspond to the original mantle layer of the developing neural tube which in the rat [1] and mouse persists into adult life. The radioactive nuclei responsible for the reactions were either resting or at various stages of mitosis (Fig. 2). Further radioactive nuclei of similar cytological appearance were also seen outside the mantle layer within 100 μ of its margin (Fig. 2).

Outside this region, radioactive nuclei were rare, but by no means absent. Most were small and dark-staining glial nuclei, found singly or as satellites of neurons. A thorough search of the sections also showed rare radioactive nuclei which were sufficiently large and light-staining to be tentatively identified as neurons (Fig. 1).

It may therefore be concluded that appreciable DNA synthesis is taking place in the mantle layer of the cerebrum, a finding which is consistent with the presence of numerous mitotic figures in this region. The next most frequent site of activity is in cytologically similar cells in the adjacent gray matter (Fig. 2, top), which fact suggests that these are actively dividing cells migrating away from the mantle layer.

Elsewhere DNA synthesis is observed in the occasional neuroglia cell and perhaps also in the rare neuron. In these cells, too, it is probably associated with mitosis, although the possibility of DNA synthesis leading to other processes (endopolyploidy?) has to be considered.

¹ This work was carried out with the support of a grant from the National Cancer Institute of Canada.



Hematoxylin-eosin stained, coated radioautographs of cerebrum from a 14 g mouse given a subcutaneous injection of 100 μ c of H^3 -thymidine and sacrificed 8 hours later. 28-day exposure.

Fig. 1.—Gray matter. In the center, an agglomeration of silver granules (arrow) is seen overlying a nucleus which seems to belong to a neuron.

Fig. 2.—Ependymal region. At the base of the picture note the lumen of the lateral ventricle (V) lined by cuboidal cells with light nuclei, the *ependyma*. Clusters of cells with somewhat darker nuclei, the *mantle layer*, surround the ependyma. In the upper half of the picture, gray matter may be seen. In addition to scattered silver granules (background fog), a number of radioactive cells are indicated by arrows. Most of them are in the mantle layer, including a prophase (P), a metaphase (M) and an anaphase (A). Two radioactive nuclei are seen within the gray matter.

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ABSENT FRUCTOSE-1,6-DIPHOSPHATASE ACTIVITY IN HEPATOMA

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DURING the past 4 years a systematic investigation has been conducted in the Montreal Cancer Institute to elucidate the major carbohydrate metabolism differences between normal and neoplastic liver [6, 14]. Enzymatic studies on glucose-6-phosphate utilization have demonstrated the following metabolic picture in the Novikoff hepatoma: normal phosphohexoseisomerase [14], absent glucose-6-phosphatase [9], markedly decreased phosphoglucomutase [12] and increased glucose-6-phosphate dehydrogenase activities [10]. The enzymatic alterations in the glucose-6-phosphate metabolism of the Novikoff hepatoma seem to be specific because no similar changes were found in regenerating, embryonic or newborn rat liver [11, 13, 14].

These enzymatic studies were recently followed up by the application of isotope technics to the investigation of carbohydrate metabolism in liver and hepatoma slices [1, 8]. The isotope work confirmed the absence of glucose release and the marked decrease in glycogen formation. The results were also in line with a possible increased shunt. An especially striking finding was the accumulation of large quantities of lactic acid in hepatoma slices.

Several factors could be responsible for the accumulation of lactic acid. One possibility is the lack of reversibility of reactions between pyruvate and glucose-6-phosphate. In the pyruvate→glucose-6-phosphate step series there is an important one-way reaction which is catalysed by fructose-1,6-diphosphatase. It was postulated that a decrease or an absence of this enzyme might explain, partially at least, the fact that almost all the glucose-6-phosphate formed in the hepatoma cell was broken down to pyruvate.

Fructose-1,6-diphosphatase activity was examined in normal liver and in Novikoff hepatoma. Male, adult Wistar rats weighing 150–200 g were used in these experiments. Purina Fox Chow and water were available *ad libitum*. The Novikoff hepatomas were transplanted intraperitoneally by using the large inoculum method [14] in-

jecting $50 \text{ million} \pm 10 \text{ million}$ cells. The tumors were 7 or 8 days old when used. Homogenates of normal liver and Novikoff hepatoma were prepared in isotonic KCl as previously described [14]. The enzymatic activity was estimated in the supernatant of normal liver and hepatoma since intracellular distribution studies showed that the fructose-1,6-diphosphatase is concentrated in this fraction [2]. The supernatant fluid was prepared by centrifuging a 10 per cent homogenate at 100,000 g for 30 minutes at 0°C. A refrigerated Spinco Model L centrifuge was used. Fructose-1,6-diphosphatase activity was estimated according to the method of Pogell and McGilvery [5] at pH 9.5 in the presence of borate buffer after 6 hours of preincubation at 38°C.

The normal liver enzymatic activity, measured by the release of inorganic phosphate at 5 and 10 minutes' incubation, was proportional with amount of enzyme added and with reaction time. Under these circumstances no fructose-1,6-diphosphatase activity was demonstrated in the Novikoff hepatoma. Incubation of hepatoma supernatant for an hour also failed to show any phosphorus liberation.

Fructose-1,6-diphosphatase activity was also measured in later studies without preincubation at pH 7.0 in the presence of glycylglycine buffer and MgSO_4 . At this pH the presence of glucose-6-phosphatase activity could interfere with the reaction. However, the supernatant fluid, when prepared by the above-mentioned centrifugation procedure, was shown previously to be free of glucose-6-phosphatase [7]. When rat liver supernatant was assayed at this physiological pH, normal enzymatic activity was found. However, the hepatoma showed no fructose-1,6-diphosphatase activity under the same assay conditions. One-hour incubation of the hepatoma supernatant with the reaction mixture also failed to result in phosphorus liberation.

To study the possible presence of an inhibitor, hepatoma supernatant was added to normal liver supernatant and the enzyme was assayed. The fructose-1,6-diphosphatase activity of the normal liver was not affected by the addition of hepatoma supernatant. This finding does not support the assumption that the presence of an inhibitor may explain the absence of fructose-1,6-diphosphatase activity in the Novikoff hepatoma. It is of interest that previous similar studies for the demonstration of an inhibitor of glucose-6-phosphatase activity in neoplastic liver were also negative [9, 14].

Krebs [3] pointed out that in the synthesis of glucose or glycogen from lactate or pyruvate, there were three energy barriers: (1) the reversal of the formation of glucose-6-phosphate from glucose and ATP, (2) the reversal of the formation of fructose-1,6-diphosphate from fructose-6-phosphate and ATP, and (3) the transfer of phosphate from phosphopyruvate to ADP. The pathway of carbohydrate synthesis is made thermodynamically feasible by circumventing the energy barriers existing at the three stages mentioned. In cases (1) and (2) the barriers are circumvented when in the synthetic pathway the phosphate of the hexosephosphate esters is removed by hydrolysis rather than by transfer to ADP. By the presence of the activity of glucose-6-phosphatase and fructose-1,6-diphosphatase the formation of glycogen and glucose from fructose-1,6-diphosphate becomes exergonic.

The absence of glucose-6-phosphatase and fructose-1,6-diphosphatase in the hepatoma, on the other hand, makes thermodynamically unfeasible the synthesis of carbohydrates from non-carbohydrate precursors. Thus, the absence of these two enzymes in the hepatoma, in the presence of normal phosphohexoseisomerase activity, favors an irreversible channelling of glucose-6-phosphate into glycolytic break-down.

In conclusion it appears that in the Novikoff hepatoma fructose-1,6-diphosphatase activity is absent or, at least, cannot be demonstrated by the usual assay methods. The absence of fructose-1,6-diphosphatase in the neoplastic liver seems to be of special importance in explaining the lack of gluconeogenic response in this tissue [1, 4, 15]. The lack of gluconeogenic response along with the decreased glycogenesis and absent glucose release will result in the irreversible channelling of the glucose molecule into pyruvate formation.

The investigation of the pathways of pyruvate utilization and the study of fructose-1,6-diphosphatase activity in regenerating, embryonic and newborn rat liver is in progress.

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EFFECT OF ADMINISTRATION OF RAT SERUM ON RAT LIVER REGENERATION¹

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BUCHER, Scott and Aub observed an increased mitotic rate in the intact liver of parabiotic rats in which the other members had been partially hepatectomized and concluded formation of a humoral growth stimulator was associated with hepatectomy [1]. Similar findings were made and conclusions drawn by Friedrich-Frekso and Zaki who injected serum from hepatectomized rats into normal rats [2]. Glinos and Gey in studying the effect of rat serum on tissue culture, and mitosis in the livers of rats which had been plasmaphoresed, concluded that serum contained an inhibitor of liver growth and that the concentration of the inhibitor was decreased

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in recently partially hepatectomized rats [3]. The present investigation was undertaken to discover the effect of administration of normal rat serum and serum from laparatomized and partially hepatectomized rats on liver regeneration following partial hepatectomy.

Male 200–230 g Holtzman rats were used. Serum was obtained from 186 rats which were divided into groups: one in which the members were partially hepatectomized according to the procedure of Higgins and Anderson [4], another in which a simple laparotomy was performed, and a third in which the members were not treated. Forty-eight hours later, during a period of maximum liver growth, the animals were anesthetized with ether and exsanguinated from a carotid artery. The sera were separated by centrifugation at 3°C and stored in aliquots at –60°C.

The rats in the experimental groups were partially hepatectomized and subsequently injected intraperitoneally with 1 ml every 12 hours, or 1.2 or 2.0 ml every 8 hours of the various sera. Control animals were treated similarly with 0.9 per cent NaCl. All the rats were given 20 per cent dextrose to drink the first 24 hours following operation. Rats in the first experiment were kept on 20 per cent dextrose an additional day, while those in the second experiment were kept on 10 per cent dextrose the second and third days. All animals were fed laboratory chow *ad lib*, except those in the third experiment which were starved for 24 hours before sacrifice. Seventy-two hours after hepatectomy, when most regeneration should have occurred, the rats were sacrificed with ether and the livers were removed and trimmed of non-hepatic tissue and necrotic liver remnant distal to the ligature. The livers were weighed to 0.01 g and the per cent liver of the total body weight at time of sacrifice was calculated for all animals. Livers were homogenized and the protein in trichloroacetic acid precipitates was determined by the method of Lowry *et al.* [5], following which the per cent protein of the livers was calculated. The animals were weighed at the beginning and the end of the experiments and per cent losses in body weight during the experiments were calculated.

Results are summarized in Table I. No differences were detected between the animals receiving 1 ml of the various preparations every 12 hours. With increased dosages both normal serum and serum from laparatomized animals had an inhibitory effect on liver regeneration which was significant at the 5 per cent level. Although there were significant differences in the fresh weights of the livers associated with differences in treatment, the per cent liver protein did not vary significantly. This suggests the differences in weight reflect true differences in amount of protein synthesized during regeneration. No significant differences were observed between animals receiving serum from laparatomized rats and those receiving serum from hepatectomized rats. Differences in some values between the three experimental groups are probably due to differences in dietary treatment. The significant loss of total body weight in the animals of experiment number 3 which were treated with saline as compared with those treated with serum was probably due to the nutritional value of the serum administered during the period of post-operative anorexia and starvation. Although serum apparently stimulated gain in total body weight in this experiment, it was seen to inhibit liver regeneration when final absolute weights of the livers were compared. Thus, the mean liver weight of animals receiving serum was 4.70 g, of those receiving saline, 5.09 g; $P = .01$.

It can be concluded that rat serum contains a factor or factors which inhibit liver

TABLE I. *Effect of serum on regenerating liver.*

Exp.	Injection schedule	Injected with	No. rats	% liver			% liver protein			% change in b weight	
				Mean	Range	P	Mean	Range	P	Mean	Range
1	1 ml every 12 hours	Saline	11	2.65	2.17-2.94	> 0.1	12.1	10.9-13.3	> 0.1	8.8	1.4-21.7
		Serum-laparot. rats	11	2.61	2.28-3.09		12.8	10.9-15.2		7.0	1.0-14.5
		Serum-hepatect. rats	9	2.49	2.23-2.91		12.5	10.1-14.8		6.9	0-14.6
2	1.2 ml every 8 hours	Saline	8	3.10	2.65-3.29	.05	10.8	10.4-11.3	> 0.1	5.4	0.9-10.0
		Serum-laparot. rats	9	2.93	2.60-3.38		10.8	9.5-12.1		6.2	2.6-10.8
		Serum-hepatect. rats	10	2.96	2.60-3.39		12.3	11.6-13.5		5.6	0.9-11.0
3	2 ml every 8 hours	Saline	10	2.94	2.82-3.28	< .05	11.4	9.4-12.7	> 0.1	19.2	13.3-23.6
		Normal rat serum	9	2.53	2.21-2.96		11.9	11.3-13.0		15.2	10.4-21.0

growth *in vivo*. Dilution of a growth stimulator by normal serum treatment is unlikely in view of the observation that saline treatment did not inhibit regeneration as serum did, and that serum from hepatectomized rats did not have less inhibitory action than normal serum. These experiments have not excluded the possibility that serum from hepatectomized rats has less inhibitory action. Possibly even though a specific inhibitor is decreased in the serum of hepatectomized rats, there is sufficient quantity, when such inhibitor is added to that already present in the animal, to cause a degree of inhibition of regeneration which could not be distinguished from that caused by normal serum. It is also possible that serum from hepatectomized rats contains less of a specific inhibitor of liver growth but contains non-specific toxic substances which have accumulated in the donor animals.

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